Association between pro-inflammatory cytokine expression, angiogenesis, extracellular matrix remodeling, and prognosis in cervical cancer

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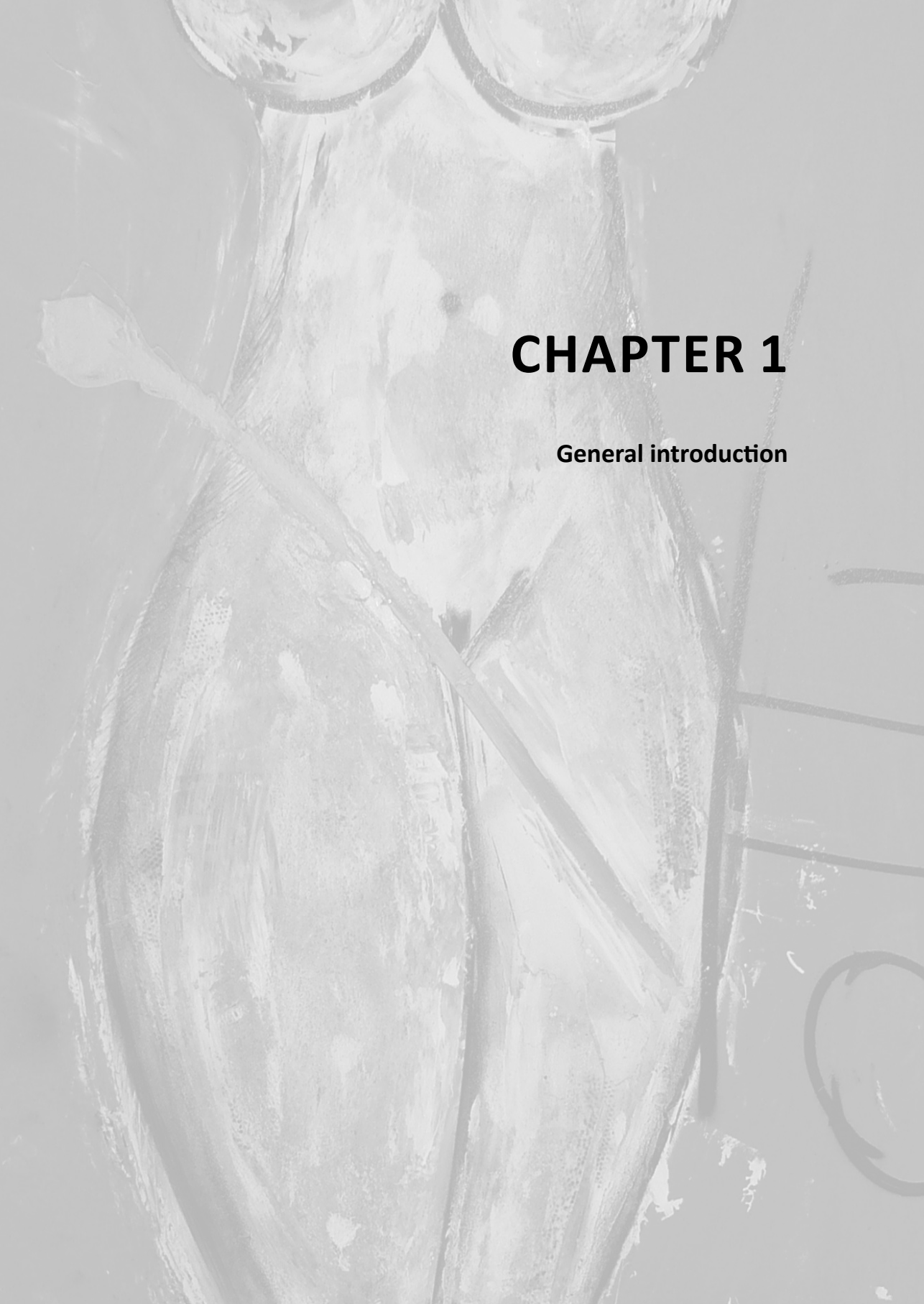

CHAPTER 1

General introduction

1 CERVICAL CANCER

Cervical cancer is the third most common female malignancy in the world with about 529,000 new cases occurring annually. More than 80% of these cases occur in developing countries where cervical cancer often ranks first place.[1] It is the second largest cause of female cancer mortality worldwide with 274,000 deaths each year. The highest incidence rates are found in South America and Africa with 37.7 per 100,000 in Brazil, 43.9 per 100,000 in Peru and 47.3 per 100,000 per year in Zimbabwe.[2]

In the Netherlands, mortality trends of cervical cancer have declined. In 2007 the incidence rate was 6.3 per 100,000 women years and mortality rate 1.4 per 100,000 women years (age adjusted).[3] In 2010, 718 new cases of cervical cancer were reported.[4]

1.1 Etiology and carcinogenesis

Epidemiologic and clinical data show that the development of cervical cancer is a multifactorial process in which infection with HPV takes a central place.[5] There are more than 150 types of HPV identified of which the mucosal types are associated predominantly with anogenital lesions. These can vary from benign warts (related to infection with HPV 6 and 11)[6] to a persistent infection with one of the high-risk HPV leading to invasive cancer. There are about 15 types of HPV classified as high-risk types. Examples of high-risk HPV types are HPV 16 and HPV 18, together responsible for 70-75% of cervical cancer.[7] Other high-risk types are types 31, 33, 35, 45, 52, 56, 58, 59, 68, 73, and 82 with different type-specific prevalence in high-grade squamous epithelial lesions (HSIL, see further this chapter) and invasive cervical cancer worldwide.[8] In 1999, Walboomers *et al* detected HPV in almost all cervical cancer biopsies, giving a prevalence of 99.7%.[9] HPV is very common and estimated is that 75% – 80% of all sexually active people will be infected with HPV at some point. 90% of these infections will be eliminated in two years.[10]

HPV is found in virtually all cervical cancers and is thought to be the causative factor in cervical cancer. Additional factors such as smoking, an early sexarche, lifetime number of sexual partners and low socioeconomic status are thought to contribute to the pathogenesis.[11]

1.2 Human papillomavirus

HPV is a causative agent in (probably) all cases of dysplasia.[12] The HPV genome consists of a double-stranded circular DNA molecule of approximately 8000 base pairs wrapped in a protein shell, called capsid. The DNA contains seven early (E) genes (E1, E2, E3, E4, E5, E6 and E7) and two late (L) genes (L1 and L2). After HPV-infection of the (basal layer of) epithelial cells of the cervix, the HPV genome is able to replicate episomally and this seems to be the situation in benign and preneoplastic lesions. In malignant lesions however, HPV DNA is frequently integrated into host DNA. This integration corresponds with the development of cervical intraepithelial neoplasia (CIN) lesions and coinciding expression level of early HPV oncogenes E5, E6, and E7.[13] These genes are responsible for initiation and progression of cervical cancer as both E6 and E7 are known for inactivation of the major tumor suppressor genes p53 and retinoblastoma protein (pRb), respectively. The role of p53 is to control the integrity of the genome by inducing cell cycle arrest or apoptosis. pRb plays an important role in controlling the correct G1/S transition of the cell cycle, a transition after cell growth (G1-phase) and before DNA synthesis and replication (S-phase). E6, in combination with host E6 associated protein (E6AP), binds and marks p53 tumor suppressor protein leading to degradation of p53 by proteasomes, genetic instability and progression to malignancy.[14] E7 competes with pRb, binding and inactivating the retinoblastoma protein by proteasomal degradation. Consequently, the transcription factor E2F (retained by pRb) is freed, stimulating the expression of genes involved in the progression of cell cycle and DNA synthesis and pushing the cell cycle forwards. Under physiological conditions free E2F leads to induction of $p16^{INK4}$, a tumor suppressor protein inhibiting cyclin-dependent kinase binding to cyclin D which regulates the G1 cycle checkpoints and causes cell cycle arrest in the G1 phase.[15] In cells infected with HPV, however, E6 blocks the inhibitory effect of $p16^{INK4}$, showing a synergistically interaction with E7 and resulting in altering cell differentiation by keeping the keratinocyte in a favorable state for viral genome replication, reactivation of DNA synthesis, stimulation of progression of the cell cycle and inhibition of apoptosis.[14, 16]

1.3 Precursor lesions of cervical cancer

The cervix consists of an ectocervix covered with stratified squamous epithelium and an endocervix, covered with simple columnar epithelium. The border between ectocervix and endocervix is the part where columnar epithelium is replaced by squamous epithelium and is known as the transformation zone. Replacing of columnar epithelium by squamous epithelium is a continuous physiological process called metaplasia. Metaplasia can occur in response to chronic physical or chemical irritation. An abnormal development of the transformation zone is called dysplasia and is characterized by an expansion of immature cells. It is considered a precancerous condition.[17]

Dysplasia, as well as cervical carcinoma, can be detected by using the Papanicolaou (Pap) test, taking a swab of the cervix (Pap smear) in a screening program or in case of complaints like irregular vaginal blood loss, discharge or vaginal bleeding after sexual intercourse. This test examines the presence of abnormal cervical cells resulting in a Pap smear classification. In 1988 an alternative reporting system has been developed for cervical cytology, called The Bethesda System, last revised in 2001. Main differences with the originally Pap classification is combining Pap results into low-grade squamous intraepithelial lesion (LSIL) and HSIL and the inclusion of specimen adequacy in order to establish uniform terminology and adequacy for evaluation.[18]

If the abnormal cells have a high chance of (becoming) cervical cancer, further investigation will be advised, leading to follow-up Pap smears after 6 months or a colposcopy with taking biopsies of abnormal areas. Biopsies can provide evidence for (pre)cancerous changes. Precancerous stages are called CIN. CIN lesions are categorized in CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia), and CIN 3 (severe dysplasia and carcinoma *in situ*), depending on the height of abnormal cells in the squamous epithelium.[19] Most CIN-lesions are stable and do not develop into cervical cancer. CIN 1 is usually treated conservative with follow-up Pap smears. The risk of progression of untreated CIN 1 to CIN 3 or (micro invasive) cervical cancer is estimated at 2.1% in 2 years time whereas about 70% of CIN 1 lesions will regress within 2 years. CIN 2 and 3 are usually treated with a large loop excision of the transformation zone (LLETZ), since progression of untreated CIN 2 to CIN 3 or (micro) invasive cervical

cancer is much higher, namely 16% in 2 years time.[20] Progression of untreated CIN 3 to cervical cancer is estimated at 31% in 10 years.[21] The transition from CIN 3 to (invasive) cervical cancer depends on whether or not the basement membrane of the epithelium is crossed and malignant cells invade the underlying stroma of the cervix.

1.4 Cervical cancer, staging and therapy

Cervical cancer is staged according to the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) staging system (specific for gynecological malignancies). Besides the FIGO staging system there is also the tumor, node, metastases (TNM) staging system which is a surgical staging and can be used for all tumors. These two systems are not always comparable and can cause confusion since both systems are used, especially when recording lymph node involvement. Cervical cancer is the only gynecological malignancy that is staged clinically according to the FIGO staging system. In the United Kingdom a survey was carried out to determine which staging system is preferred and FIGO staging was recommended. TNM style recording of lymph node status was also recommended since lymph node status is not included in FIGO staging.[22] Recently, both staging systems were revised, based on clinical and histomorphological studies.[23] Cervical cancer spreads predominantly by local invasion to the vagina and parametrium and by lymphatic metastasis to pelvic lymph nodes. Pelvic lymph node metastases are found in 0% - 88% of the patients depending on FIGO stage.[24] Para-aortic lymph nodes containing metastases are secondary stations and are detected in 15-30% of the patients with locally advanced cervical cancer.[25] Hematogenous spread is detected in combination with bulky disease, endometrial extension and lymph node metastasis. The most common sites are lungs (present in 3% - 57% depending on FIGO stage)[26] and bone (1.9% - 16%).[27]

Surgical treatment relies on FIGO stage and can vary from conization to a radical hysterectomy with removal of the tissue on both sides of the uterus (parametrium), the upper part of the vagina and pelvic lymph nodes (external iliac, common iliac and obturator fossa). In case of larger early tumors (stage IB2 and IIA2) radiotherapy (with or without chemotherapy) or an extended surgical procedure can be chosen (Wertheim Okabayashi). In case of advanced stage tumors (stage IIB to IVA) radiotherapy with or without chemotherapy is the treatment of choice.

2 INTERACTION BETWEEN THE IMMUNE SYSTEM AND CERVICAL CANCER

2.1 Inflammatory response

Most neoplastic tissues have an inflammatory component consisting of a diversity of inflammatory cells. This inflammatory component of the tumor can be initiated by predisposing conditions to cancer, but also by genetic events causing neoplastic transformation.[28] Cells forming the cancer-related inflammation (CRI) are for instance leukocytes, dendritic cells (DC) and tumor associated macrophages (TAM), but also myofibroblasts, mast cells and to a lesser extend natural killer (NK) cells.[29] In this process, the tumor microenvironment (including fibroblasts and vessels) is the result of the interaction between tumor cells, stromal cells and inflammatory cells influencing tumor growth, tumor differentiation and tumor progression. Both tumor cells as well as cells of the tumor environment are capable of producing soluble mediators like cytokines, of which the balance can lead to antitumor activity or tumor progression.[30]

Antitumor activity is mainly mediated by circulating effector cells, in particular cytotoxic T cells, NK cells and neutrophils.[31] Also adaptive immunity (B- and T-lymphocytes) can be efficient as an antitumor response, generating memory cells and being more specific.[32] Several studies show a positive correlation between poor outcome and composition of the tumor environment with a special role for angiogenesis and TAM with tumor hypoxia and secondary necrosis due to rapid tumor cell proliferation as a possible driving force for the production of growth factors and chemoattractive cytokines like chemokine C-C motif ligand (CCL)2.[33] TAM are frequently present in the tumor environment and are thought to be important in cross-talk with other inflammatory cells and cancer cells. They are derived from monocytes circulating in the blood and attracted to the tumor by specific produced chemotactic factors like CCL2.[34] Once in the tumor stroma, monocytes can differentiate into a M1 macrophage (an effector cell known for killing microorganisms and tumor cells and supplier of proinflammatory cytokines) or into a M2 macrophage (cells capable of tuning the inflammatory responses, scavenging debris, promoting wound healing, angiogenesis and tissue remodeling) (Figure 1).[35] TAM are known for their diversity of released growth factors leading to tumor progression and metastases.[36, 37]

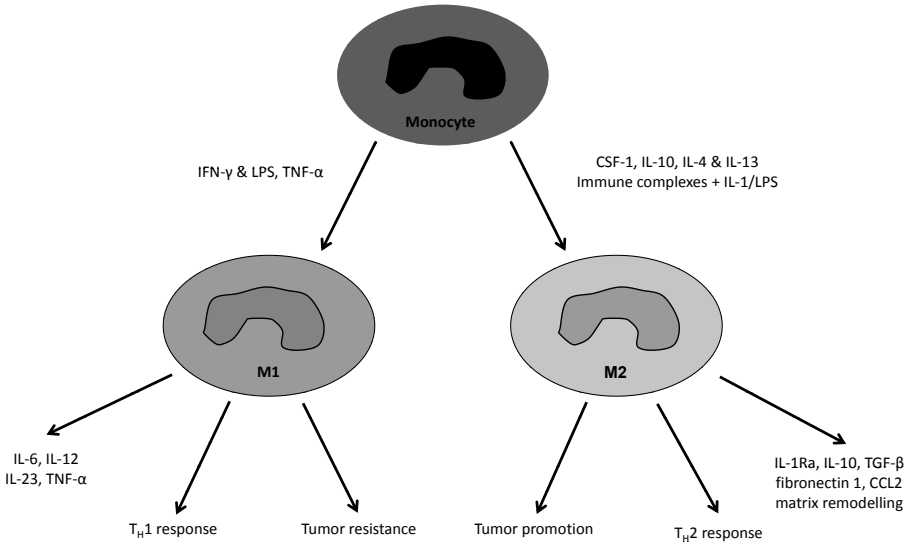

Figure 1. Selected functions of M1 and M2 macrophages. Monocytes are attracted to the tumor stroma by tumor derived signals. Once in the tumor stroma, monocytes are able to differentiate into M1 or M2 macrophages, depending on different microenvironmental signals. Abbreviations: CSF-1, colony stimulating factor-1; IFN-*γ*, interferon-*γ*; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; T_H, T helper; TGF-β_1, transforming growth factor-β_1; TNF, tumor necrosis factor.

They play a major role in this process by providing pro-angiogenic cytokines, tumor stroma remodeling factors (for instance matrix metalloproteinases (MMP)[38] and growth factors thus providing an ideal basis for angiogenesis (Figure 2). Monocytes are also capable of differentiating into (inflammatory) DC.[39] DC are one of the most potent antigen-presenting cells of the immune system and form a heterogeneous group of cells originating from different backgrounds. They can be classified into two major subsets: (1) the myeloid DC (mDC), containing three types of DC: the epithelial DC (Langerhans' cells (LHC), almost exclusively found in epithelia), the interstitial DC (found in the interstitium and extravascular areas of most tissues) and the monocyte-derived DC (blood myeloid DC), and (2) the lymphoid DC (lDC) subset, cluster of differentiation (CD)11c$^-$ plasmacytoid DC precursors, present in blood, tonsils and thymus). Interstitial and monocyte-derived DC display phenotypic and functional similarities.[40, 41] DC display different molecules and receptors on the surface to bind and take up foreign material or debris which is processed.

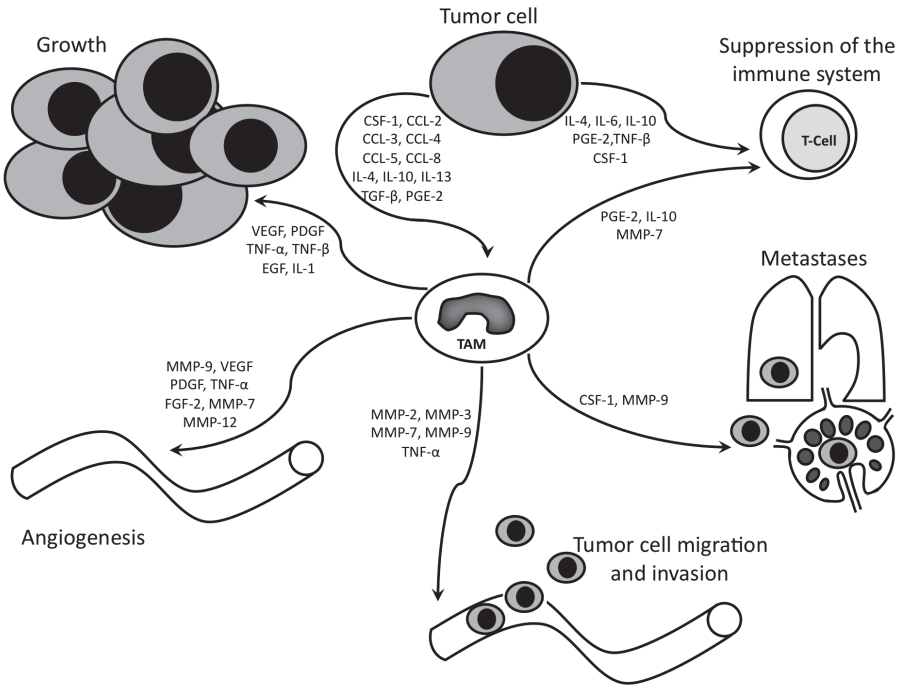

Figure 2. Role of tumor associated macrophages in tumor progression. Abbreviations: CCL, chemokine (C-C motif) ligand; CSF-1, colony stimulating factor-1; EGF, epidermal growth factor; FGF, fibroblast growth factor; IL, interleukin; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PGE-2, prostaglandin E2; TGF-β_1, transforming growth factor-β_1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
Adapted from Rogers et al.[95]

After maturation of the mDC, in response to stimuli like tumor necrosis factor (TNF)-α, these cells migrate from peripheral tissues via the lymph vessels to T and B cell areas of secondary lymphoid organs. Here, processed antigens, in major histocompatibility complex molecules (MHC) class I or II and co-stimulatory molecules like CD80 and CD86, are presented to naive $CD8^+$ and $CD4^+$ T cells, subsequently triggering an adaptive immune response.[42, 43] Within the lymph nodes, DC and LHC seem to migrate to distinctive locations: DC cluster nearer to the B cell follicles, probably more involved in regulation of the humoral responses, while the LHC move to the deep paracortex of the T cell zone, suggesting that LHC are more involved in a T-cell immune response.[44] Previously it was assumed that the immune response depends

on the type of subset of DC in which the mDC induce a T helper lymphocyte 1 (T_H1) and the lDC a T helper lymphocyte 2 (T_H2) response. In general this still seems to be true, but some refinement has been made. Especially the mDC are capable of activating both a T_H1 and/or a T_H2 response dependent on the presence of various maturation stimuli.[45] Normal DC are capable in inducing a T_H2 response after contact with an allergen. In case of an infection, the combination of pathogen and allergen can differentiate DC either into a DC1 subtype, activating the T_H1 system or into a regulatory DC (DCreg) subtype, inducing a regulatory T cell (Treg) response.[46] T_H1, T_H2 and also T helper 17 (T_H17) are all effector T-cell subsets with different functions and with a different profile of produced cytokines (Figure 3). In general T_H1 cells are important for host defense against intracellular pathogens and involved in induction of delayed-type hypersensitivity responses. T_H1 response is considered to be pro-inflammatory. T_H2 cells play an important role in eosinophilic inflammation, allergic reactions and clearing extracellular organisms and is linked to immunoregulatory or anti-inflammatory and promotes antibody-mediated immunity.[47] T_H17 cells are mainly involved in organ-specific autoimmunity, mediating host defenses and promoting tissue inflammation and recruitment of neutrophils. At present, their role in cancer is still unclear. Regulatory T cells (Treg) play an important role in controlling autoimmune pathogenesis.[48] Furthermore, FoxP3 positive Treg seem to regulate the immune response in cervical cancer.[49]

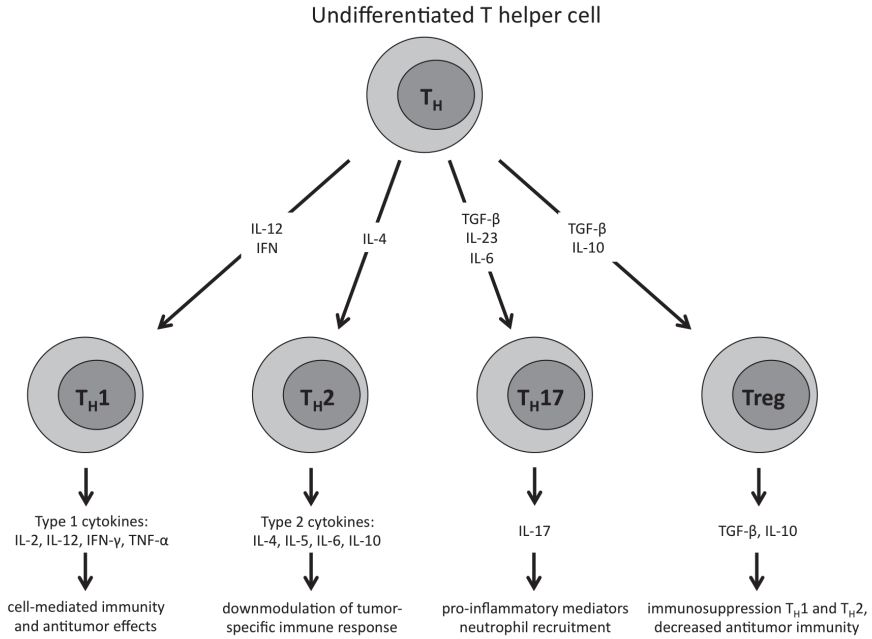

Figure 3. Example of differentiation of T helper lymphocyte subsets by cytokines. Abbreviations: IFN, interferon; IL, interleukin; TGF-β_1, transforming growth factor-β_1; T_H, T helper cell; TNF-α, tumor necrosis factor-α; Treg, regulatory T cell.

2.2 Cytokines and chemokines

Cytokines are small proteins and peptides, secreted by different cell types in nano- to picomolar concentrations. Cytokines transmit signals between cells locally and at a distance in different ways: autocrine (produced cytokines acts on the cytokine-producing cells), paracrine (produced cytokines acts on near-by cells), intracrine (after internalization of extracellular signaling proteins and peptides, these proteins and peptides interact with intracellular targets),[50] juxtacrine (membrane-bound cytokines produced by an effector cell acts via a membrane-bound cytokine receptor on a adjacent target cell) or retrocrine (soluble forms of normally membrane-bound receptors are produced and reacts with distant target cells) (Figure 4). Recently, also the term necrocrine was introduced, aiming at the effect of cytokines released by necrotic cells.[51] Cytokines are active in virtually every aspect of immunity and inflammation and determine whether an immune response will develop and how this response will be: cytotoxic, humoral or cell-mediated.[52]

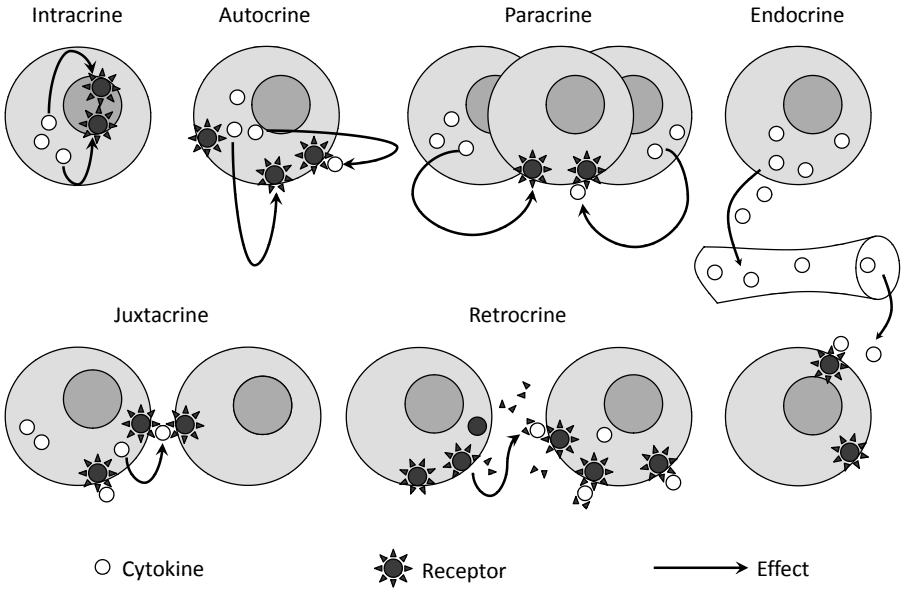

Figure 4. Possible ways of transmitting signals between cells by cytokines: intracrine (produced cytokine acts inside the producing cell), autocrine (secreted cytokine binds to receptors on the same cell), paracrine (secreted cytokine binds to receptors on a cell nearby), endocrine (cytokine is secreted directly into the bloodstream), juxtacrine (intercellular communication between adjacent cells by both binding secreted cytokine) and suggested retrocrine factors (soluble parts of receptors interact with target cells by binding to cytokines which are membrane-bound on this target cell).
Adapted from Simpson et al.[96]

Cytokines can be divided into subcategories, depending on type or function, for example pro-inflammatory cytokines [TNF-α, Interleukin (IL)-12, IL-6], growth factors [granulocyte colony-stimulating factor (G-CSF), IL-2] or inhibitory cytokines [IL-10 or transforming growth factor (TGF)-β_1] (Table 1). Chemokines are a group of small molecules, structurally related to cytokines with chemotaxis (cellular directional movement on the basis of a concentration gradient of chemotactic factors) as most important effect. They regulate the activity by interacting with guanine nucleotide binding (G-) protein-coupled 7-transmembrane receptors. In humans they can be divided in α-chemokines, genes of this family map to human chromosome 4q12-21 and share a separation of the first two cysteine residues by a single amino acid [CXC (motif) –chemokines]; β-chemokines, genes map to chromosome 17q11-32 and in these chemokines the first two cysteine residues are adjacent (CC-

chemokines); *γ*-chemokines or XC (motif) -chemokines which lack a cysteine residue and *δ*-chemokine (CX3C-chemokine) which is a type 1 transmembrane glycoprotein (Table 2).[53]

Cytokines act as part of a complex network in which redundancy (several produced cytokines display similar properties leading to additive effects, for example IL-2, IL-4, IL-7, IL-9 and IL-15 which all can act as T-cell growth factors) and pleiotropism (many cytokines are able to have an effect on different cell types leading to different consequences). An example of pleiotropism is IL-2 which is able to induce proliferation of T cells, but also cytolytic activity of NK cells and immunoglobulin biosynthesis by B-cells.[54] Cytokines can have dual roles depending on the cell type, for example interferon (IFN)-*γ* which promotes T_H1 responses, also inhibits development of a T_H2 response. Cytokines act by binding to a receptor on the surface of an effector cell after which signal transduction molecules carry the signal from the receptor to the inside of the cell or nucleus leading to transcription of specific genes. Examples are IL-6 and IL-12. Both are bound by specific receptors on the surface of the effector cell, activating Janus kinases (JAK) in the cytoplasm. Subsequently, signal transducer and activator of transcription (STAT) molecules are phosphorylated and migrate to the nucleus.[55]

Chemokines are bound by a specific transmembrane G-protein-coupled receptor and after activation, an intracellular signaling cascade is started, causing migration of the target cell towards a high concentration of the chemokine. A good example of this process is reflected by the immunosurveillance of leukocytes. Normally these cells circulate in the blood vessels but under the influence of, for example, CCL2 they move to the interstitial compartment: first by tethering and rolling on the surface of the endothelial cells (a process mediated by selectins) followed by activation, arrest and transmigration to the interstitial compartment where the highest concentration of chemokine is present.[56]

Table 1. Characteristics of cytokines and chemokines investigated in this thesis.

Name	Family	Source	Receptor	Signal transduction	Function	Prominent in	Stimulating factors	Inhibiting factors
CCL2	CC chemokines	Endothelial cells, fibroblasts, monocytes, macrophages, DC, smooth muscle cells, mesangial cells, osteoblastic cells	CCR2	Ca NF-κB	Recruiting leukocytes to sites of infection, trauma or inflammation Involved in angiogenesis Autocrine growth factor	Colorectal Breast Stomach Cervix	Oxidative stress Growth factors Pro-inflammatory cytokines Lipopolysaccharides IL-1 IgG complexes	Eotaxin
IL-6	Interleukin type I, IL-6 like/gp130	Monocytes, macrophages, fibroblasts, endothelial cells, T cells, B lymphocytes, granulocytes, smooth muscle cells, eosinophils, keratinocytes	IL-6R	JAK-STAT pathway, PI3K, MAPK pathway	Cell differentiation factor. Growth factor. Immune regulator. Involvement in the balance between T_H17 and Treg	Cervix Colorectal Bladder	Viral infection, IL-1, IL-17, TNF-α, PDGF, GM-CSF, LPS, IFN-β Tissue damage, endotoxins	Estrogen Gluco-corticoids IL-4
IL-12	Interleukin type I, IL-12 family	DC, monocytes, macrophages, keratinocytes, lymphocytes	IL-12R complex	JAK-STAT pathway	Differentiation and stimulation of naïve T cells to T_H1 cells Stimulation TNF-α production by T cells and NK Enhancing cytotoxic activity of $CD8^+$ T cells Activating innate and adaptive immunity	Head and neck Cervix B cell non-Hodgkin's lymphoma	Bacterial products parasites IL-4, IL-13 IFN-γ	IL-12p40, IL-10 TGF-β
IL-17	Interleukin 17 family	T_H17 cells		RORγt, NFAT, STAT3?	Tumor growth Angiogenesis	Ovarian Breast Prostate Lung	Bacterial products	IL-4 IFN-γ

Name	Family	Source	Receptor	Signal transduction	Function	Prominent in	Stimulating factors	Inhibiting factors
IL-23	Interleukin type I IL-12 family	Keratinocytes, macrophages, DC	IL-23R complex	STAT3	Promoting inflammatory response Angiogenesis Proliferation and expansion of T_H17 cells	Colon Ovarian Head and neck Lung melanoma	Microorganisms, CD40L in combination with TLR ligand PGE_2	IFN-γ IL-10
GM-CSF	Interleukin type I, β-chain family	Macrophages, T cells, fibroblasts, endothelial cells, smooth muscle cells	GM-CSF receptor	JAK2	Production, activation and differentiation of hemopoietic cells	Breast Colorectal	Inflammation, Tissue damage, IL-1, TNF-α, LPS	
TNF-α	TNF	Monocytes, macrophages, neutrophils, T-cells, NK cells, keratinocytes, tumor cells	TNFR1 TNFR2	Caspase 8 and 3 TRAF2 – NF-κB TRAF2– NF-κB	Apoptosis Angiogenesis Activation growth factors Inflammatory response Proliferation and survival tumor cells	Melanoma Ovarian Pancreas	IFN-γ IL-2 GM-CSF	IL-6 TGF-β

Table 2. Characteristics of the different chemokine-families.

Chemokine family	Characteristic	Chromosome	Example	Function
α	-CXC-	4q12-21	CXCL8 (IL-8)	Primarily acting on neutrophils
β	-CC-	17q11-32	CCL2 (MCP-1)	Chemotactic for monocytes, role in antiviral host defense
γ	-XC-	1q23-25	XCL1 (Lymphotactin)	Chemotactic for lymphocytes and NK cells, active against bacteria
δ	-CX3C-	16q13	CX3CL1 (Fractalkine)	Chemotactic for neutrophils

2.3 Cytokines, chemokines, inflammatory cells, and cervical cancer

Cytokines are known to be produced by a wide variety of cells, including epithelial cells, stromal cells as well as cells of the immune system.[57-59] Also cancer cells and cells of the tumor environment are capable of producing cytokines with a wide variety of effects.[53, 60] Several types of cancers develop after years of chronic inflammation. Examples are lung cancer (cigarette smoking), cancer of the esophagus (gastro esophageal reflux) and cervical cancer (HPV infection). A chronic inflammatory state, together with the permanent presence of HPV could increase epithelial cell turnover resulting in DNA changes and, finally, in malignant transformation.[47] Persistent HPV infection also has its effect on the composition of cytokines.[61] Cytokine levels can be affected by malignant transformation of the cervix, as in early stage cervical cancer LHC as well as mDC numbers are found in reduced numbers[61, 62] Due to the inhibitory effect of E6 and E7 proteins, cytokines like granulocyte-monocyte colony-stimulating factor (GM-CSF), TNF-α,[63] IL-8 (*in vitro),*[64] and CCL2[65] are suppressed. In HPV-related VIN several cytokines and chemokines showed a different expression pattern when compared to control samples [e.g. up-regulation of CXCL8 (IL-8)] in combination with an increased number of mature dendritic cells in the dermis, suggesting most mature dendritic cells do not receive the appropriate (chemokine) signal to migrate.[66] Since DC are one of the most potent antigen-presenting cells in draining lymph nodes, this process may be hampered by the low number of DC.[67] The normal uterine cervix contains a limited number of different leukocytes (T lymphocytes, DC, macrophages). In cervical cancer, the lesions contain much higher numbers of $CD4^+$ and $CD8^+$ T cells as

well as Treg cells, although the latter ones are more observed in early phase of tumor progression.[68] Tumor-infiltrating immune cells are linked to prognosis and response to (conventional) therapy. High numbers of $CD8^+$ T cells are frequently correlated with a better prognosis in solid tumors.[69-71] Also a high $CD4^+/CD8^+$ T cells is of importance for a better outcome of patients suffering from cervical carcinoma.[72] A known T-cell chemoattractant is the chemokine CXCL12 which binds selectively the receptor CXCR4. In a study of Jaafar *et al* it was shown that CXCL12 could attract T cells at low concentrations and shows the opposite effect at high concentration.[73] Furthermore, balance of T cells seems to reflect tumor stage as found in both breast cancer as gastric cancer.[74, 75] In an early stage both tumors show an increased ratio of T_H17/Treg cells whereas in advanced stage the number of T_H17 cells gradually decreased while the number of Treg cells increased. In cervical carcinoma numbers of both T_H17 cells and Treg cells increase gradually with disease progression, resulting in an increased T_H17/Treg ratio.[76] The role of T_H17 is still controversial with properties either inhibiting or stimulating tumor growth and progression.[77, 78] At present, the role of T_H17 in cancer is still not clear. Treg play an important role in controlling autoimmune pathogenesis[48] and in inhibiting antitumor responses (Figure 3).[79] Furthermore, a low $CD8^+$/Treg ratio is a significant independent unfavorable prognostic factor in cervical cancer patients.[49]

3 TUMOR MICROENVIRONMENT

3.1 Extracellular matrix

Tumor cells are dependent for their function on the extracellular matrix (ECM), a component of the tumor stroma. The ECM consists (among others) of fibers of collagen, fibronectin and elastin together with proteoglycans (glycosaminoglycans) and polysaccharides (hyaluronic acid).[80] The ECM is produced by resident cells, already present in the ECM. When produced, the ECM displays several functions. From providing structural support to the cells, allowing trafficking of cells, homeostasis, to serving as a reservoir for cytokines and growth factors.[81] The cells within the microenvironment have the ability to remodel the ECM through altering the production of ECM components or producing proteases, such as MMP, thus changing

interactions with other cells or the matrix.[82] For cancer cells to survive, grow and metastasize, a unique microenvironment has to be established in which cancer cells, cancer associated stromal fibroblasts (CAF), endothelial cells, inflammatory cells and ECM are functionally organized.[83] CAF are known to promote tumor growth and invasion, probably by secreting more growth promoting factors and up-regulation of genes responsible for extracellular matrix remodeling.[83] Besides the CAF, more tissue elements may affect tumor formation and behavior. One of the components with the ability to alter cell function is versican, a member of the chondroitin sulphate proteoglycan (CSPG) family. Versican plays diverse roles in adhesion, proliferation and migration of cells, all important processes in invasion or metastasis of cancer cells. Indeed, in most malignancies elevated levels of versican have been found.[84] Versican expression correlates with a poor prognosis in cervical cancer, ovarian cancer and gastric gastrointestinal stromal tumors.[82, 85, 86] (Cervical) cancer is often infiltrated with a large amount of leukocytes which respond to tumor cells by secreting growth factors, cytokines and chemokines.[33]

3.2 Angiogenesis

ECM also provides a basis for blood- and lymphatic vessels. Tumor growth beyond a few millimeters and metastasis formation are processes, highly dependent on induction of angiogenesis. The interaction between cancer cells and tumor fibroblasts triggers an angiogenic switch in fibroblasts in experimental tumors.[87] New blood vessels are formed to ensure that tumor cells are provided with oxygen and nutrients.[88] Inflammatory cells in the tumor stroma play a major role in angiogenesis, possibly acting as initiator of tumor neovascularization by production of angiogenic factors.[89] One of the strongest angiogenic factors is vascular endothelial growth factor (VEGF), a potent mitogen, which stimulates endothelial cells to proliferate and migrate. This is achieved by binding VEGF to the VEGF receptor2 (VEGFR2) after which a strong tyrosine kinase activity is induced, leading to signals for stimulation of vascular endothelial cell survival and growth as well as promotion of angiogenesis via the mitogen-associated protein kinase (MAPK) pathway.[90] New blood vessels are formed either by sprouting of local blood vessels (angiogenesis) or de novo by *in situ* differentiation of primitive progenitors into mature endothelial cells. In tumors

both mechanisms are operative and take place concomitantly.[91] Neovascularization is possible after degradation of the ECM by proteases. Matrix metalloproteinases represent a family of proteinases which is associated with proteolysis of a variety of substrates and play an important role in this process.[92] Besides having a role in degradation of ECM macromolecules (proteoglycans and collagens), particular MMP are also capable of cleaving connective tissue growth factor (CTGF) such as VEGF, thereby releasing its angiogenic activity.[93] New blood vessels are often leaky, dilated and tortuous due to lack of the pericyte cover. Lymphatic endothelial cells have even less developed junctions with frequently large interendothelial gaps and impaired basement membranes, leading to easier penetration by tumor cells and metastasis formation.[94]

4 OUTLINE OF THIS THESIS

The aim of this thesis was to investigate the influence of (pro-inflammatory) cytokines, produced by cervical cancer cells, on the tumor microenvironment (e.g. composition of inflammatory cells, angiogenesis and extracellular matrix alterations) and its association with clinical parameters like lymph node metastases and survival in cervical carcinoma.

Chapter 2 concentrates on the chemokine *CCL2*, the expression by cervical cancer cells and its association with TAM in the tumor. CCL2 is thought to be involved in the attraction of monocytes to the tumor site where these monocytes differentiate into TAM. TAM are able to produce various cytokines, possibly leading to tumor progression. *CCL2* mRNA expression level was investigated by RNA-*in situ* hybridization (RISH). The immunohistochemical scoring method for the ISH used in this chapter was validated by a real-time quantitative polymerase chain reaction (RT-PCR) technique. Expression level of *CCL2* was divided in 3 categories: absent, low and high expression level. The samples showing a lack of expression of *CCL2* were investigated for loss of heterozygosity in the region where the *CCL2* gene is located on chromosome 17. Besides TAM, several types of antigen presenting cells are present in the tumor microenvironment. The activation, differentiation and migration of these cells by *GM-CSF*, *TNF-α* and *IL-12* was investigated in **Chapter 3**. *GM-CSF*, *TNF-α* and *IL-12*

were also detected by RISH to determine the cellular location of these cytokines. *GM-CSF* as well as *TNF-α*, expressed by cervical carcinoma cells, were proposed to play a role in the attraction of inflammatory cells to the tumor site, the differentiation of inflammatory cells and (in the case of *TNF-α*) influencing the migration of LHC from the tumor site. The expression level of *IL-12p40* was furthermore investigated in relation to clinicopathological parameters. The IL-12 family consists of several members of which IL-12 and IL-23 share the IL-12p40 subchain. In **Chapter 4** the role of *IL-12p40* was further investigated by examining the expression level of *IL-12p40* as well as *IL-23p19*, the specific subchain for IL-23. IL-12 is thought to induce an antitumor immune response whereas endogenous IL-23 may promote tumor progression. Since IL-23 plays an important role in the IL-17/IL-23 pathway leading to expansion and maintenance of T_H17 cells, tissue sections were also investigated for the presence and location of IL-17 producing cells. The IL-17/IL-23 pathway is induced by IL-1 and IL-6 and both cytokines are thought to play an important role in the induction of T_H17 cells in humans. Expression of both cytokines was investigated. Tumor growth is dependent on the supply of oxygen and nutrition. Angiogenesis has a key role in this process and many factors contribute to the formation of new blood vessels. In **Chapter 5** different factors associated with angiogenesis were investigated of which VEGF-A seems to be the most important one. Newly formed blood vessels (CD105 positive cells) were stained to differentiate these vessels from vessels already present in the tumor stroma (CD31 positive cells) and the location of the vessels in the tumor was determined. Other factors important in angiogenesis are those capable of modulating the microenvironment, clearing the way for tumor expansion. One of these factors is Matrilysin (MMP-7) which was investigated in **Chapter 6**. MMP-7 can be up-regulated by different mechanisms, hypoxia in later tumor stages being one of them. MMP-7 is not only produced by epithelial cells but also by endothelial cells within the tumor stroma. Presence of MMP-7 was compared with the presence of CD34 (a pan-endothelial marker) *in vivo* and sprout formation *in vitro.* Besides having an effect on membrane-adhering properties, MMP-7 is also capable of releasing/cleaving bioactive molecules. **Chapter 7** focuses on other alterations in the extracellular matrix (e.g. versican). Versican is a member of chrondroitin sulfate proteoglycan family. Four isoforms are known, each isoform being involved

in different biological processes. Versican expression is related to tumor growth by hampering cell adhesion and regulating angiogenesis. The expression of versican was investigated in relation to the number of inflammatory cells and relevant clinical parameters. **Chapter 8** summarizes the results and outlines future perspectives.

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CHAPTER 2

The absence of *CCL2* expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11.2

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ABSTRACT

To determine whether *CCL2* mRNA expression is beneficial or detrimental for cervical cancer patients, the association between the expression of this molecule by cervical tumor cells, the number of tumor-associated macrophages (TAM), and clinicopathological parameters such as recurrence, relapse-free survival, and overall patient survival was investigated. In cervical cancer samples from 93 untreated cervical cancer patients, the *CCL2* mRNA expression level was quantified using RNA-*in situ* hybridization and verified using real-time quantitative RT-PCR. The number of TAM was determined using immunohistochemistry. Furthermore, the study investigated whether lack of *CCL2* expression was due to genetic alterations near the 17q11.2 (*CCL2* genomic) region. *CCL2* mRNA expression by cervical tumor cells was associated with the number of TAM ($P<0.001$). Lack of *CCL2* mRNA expression (15 samples; 16%) was associated with increased cumulative relapse-free survival (log-rank test, $P=0.030$), increased cumulative overall survival (log-rank test, $P=0.024$), reduced local and distant recurrence, reduced vascular invasion, and smaller tumor size (<40 mm). The absence of *CCL2* mRNA expression corresponded with loss of heterozygosity (LOH) at 17q11.2 in five of six samples. The increased cumulative relapse-free survival and cumulative overall survival of cervical cancer patients lacking tumor cell-associated *CCL2* mRNA suggest that the TAM support tumor progression, presumably by promoting angiogenesis and production of growth-factors.

INTRODUCTION

Cervical carcinoma is an important health problem, being the second most common cancer among women worldwide and ranking first in many developing countries.[1] Infection with human papillomavirus (HPV) is the key factor in the development of cervical intraepithelial neoplasia and invasive cervical carcinoma.[1, 2] Four HPV types (16, 18, 31 and 45) account for almost 75 – 80% of the invasive cancers and HPV DNA is present in more than 90% of premalignant and malignant squamous lesions of the uterine cervix.[1, 2] Cervical tumor cells are able to impede an effective immune response. Different immune escape mechanisms have been observed. Cervical cancer cells down-regulate human leukocyte antigen (HLA)[3, 4], express FAS-ligand[5, 6] or protease inhibitor (PI)-9[7] and have been shown to produce immunoregulatory cytokines.[8, 9]

At present, the presence of an inflammatory infiltrate and the production of immunomodulatory cytokines by cancer cells is thought to contribute to carcinogenesis.[10-12] Previously, we observed that *IL-1α* and *IL-1β*, *IL-8*, *IL-12p35*, *IL-15*, *TGF-β$_1$* and *CCL2* mRNA was produced by at least nine of ten cervical cancer cell lines. In contrast to the other cytokines, *CCL2* was produced in only 30% of normal primary cervical epithelial cell cultures,[8] suggesting up-regulation of *CCL2* in cervical cancer cell lines. CCL2 is a well-known chemo-attractant for human monocytes, memory T cells, and natural killer (NK) cells.[13, 14] Furthermore, CCL2 was shown to be involved in the attraction of monocytes to the tumor site.[15-19] The attracted monocytes differentiate into tumor-associated macrophages (TAM) at the tumor site[13, 20, 21] and are thought to produce various cytokines that promote tumor progression.[20-22] An association between TAM and microvascular density or angiogenesis was often observed.[15, 23-25] Although the association between *CCL2* expression by tumor cells and the recruitment of monocytes to the tumor site is becoming increasingly clear, the relationship between expression of *CCL2* by tumor cells and clinical parameters such as positive lymph nodes, relapse or survival is unclear. Only a limited number of studies, including non-small cell lung cancer, breast cancer, esophageal squamous cell cancer, gastric cancer, and pancreatic cancer, have addressed the association between tumor-tissue derived *CCL2* expression and clinical parameters.[15, 17-19, 25, 26]

The aim of the present study was to determine whether *CCL2* mRNA expression by cervical tumor cells, resulting in the attraction of monocytes that subsequently differentiate into TAM, is beneficial or detrimental for cervical cancer patients. For this purpose, we have investigated the association between *CCL2* expression by cervical tumor cells, the number of TAM and clinicopathological parameters such as relapse free-survival and overall survival of cervical carcinoma patients. Furthermore, we have investigated whether the lack of *CCL2* expression was due to genetic alterations near the 17q11.2 (*CCL2* genomic) region.

MATERIAL AND METHODS

Patient material

Between 1985 and 1995, 254 untreated patients suffering from FIGO (Fédération Internationale des Gynécologie et d'Obstétrique) stage IB and IIA primary cervical carcinoma underwent a radical hysterectomy type III with lymphadenectomy. Of the tissue samples available, 93 were randomly chosen. Tissues were routinely embedded in paraffin wax after 10% formalin fixation. The tissue samples from each patient were examined by a pathologist for the presence of tumor. Of the 93 samples, 80 were squamous cell carcinomas, seven adenocarcinomas and six adenosquamous carcinomas. Of the 93 samples, 70 (75%) were classified as FIGO stage IB and 23 (25%) as FIGO stage IIA. Human tissue samples were used according to the guidelines of the Ethics Committee of the Leiden University Medical Center.

Preparation *CCL2* RNA-probe

For the *CCL2* RNA probe, *E. coli* HB101 containing a pGEM®-7Zf(+) vector (Promega, Madison, WI) inserted with CCL2 DNA (ATCC, Manassas, VA) was used.[27] Plasmids were isolated using the QIAfilter Maxi KITS protocol (Qiagen, Hilden, Germany). The sequence of the PCR product was confirmed by DNA sequencing. The recombinant plasmids were linearized with either EcoR I or BamH I (both Boehringer, Mannheim, Germany). Both strands were translated in a digoxigenin (DIG)-labeled RNA probe according to the manufacturer's instructions (Boehringer). The concentrations of the DIG-labeled sense and antisense RNA probes were determined on a 1% agarose gel

stained with ethidium bromide (Sigma, St. Louis, MO). Probes were stored at -20˚C until further use.

RNA-*in situ* hybridization

RNA-*in situ* hybridization (RISH) was performed as previously described.[27] In short, 3 μm thick paraffin-embedded sections were pretreated and hybridized with 100 ng/ml DIG-labeled RNA probe diluted in hybridization mixture containing 0.3 M NaCl, 0.03 M saline-sodium citrate (2x SSC). Hybridization was allowed for 16 h at 50˚C in a humidified chamber. Slides were washed for 30 min in 50% formamide/2x SSC at 42˚C; for 45 min in 0.1x SSC with 20 mM β-mercaptoethanol (Merck, Darmstadt, Germany) at 50˚C; and for 30 min with 2 U/ml ribonuclease (RNase) T1 (Boehringer) in 2x SSC, 1 mM EDTA at 37˚C. RNA hybrids were detected using mouse anti-digoxigenin (1:2000, Sigma-Aldrich Chemie, Steinham, Germany) followed by rabbit anti-mouse Ig (1:50; DAKO, Glostrup, Denmark) and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP; DAKO).[28]

Immunohistochemical staining

Serial sections of formalin-fixed, paraffin-embedded cervical carcinoma, 3 μm thick, were mounted on aminopropylethoxysilane-coated slides. Sections were dewaxed and rehydrated. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 20 min. After rinsing the sections in phosphate-buffered saline (PBS), antigen retrieval was performed by treating the sections for 20 min at 37°C with 0.1% trypsin (Sigma-Aldrich Chemie), 0.1% $CaCl_2$, pH 7.4. Sections were incubated with PBA [PBS containing 1% bovine serum albumin (BSA) (Sigma)], followed by an overnight incubation at room temperature with monoclonal antibody (MAb) directed against CD68 (1:1600, IgG1; DAKO). Subsequently, immunohistochemical staining was performed as previously described.[27]

Semi-quantitative evaluation of immunohistochemical and RISH results

The staining of *CCL2* in the tumor cells was scored as previously described.[29] Intensity was scored as none (0), mild (1), moderate (2) or intense (3) at low magnification (x100). Furthermore, the percentage of positive tumor cells was determined and

divided into groups (from 0 to 5): 0% (0, absent); 1% to 5% (1, sporadic); 6% to 25% (2, local): 26% to 50% (3, occasional); 51% to 75% (4, majority) and 76% to 100% (5, large majority). The two parameters were combined, representing the sum of both the percentage and the staining intensity of the positive cells, resulting in an overall score (0 or 2-8). The scores were combined into three groups: category 0 (score 0, no expression); category 1 (scores 2, 3, 4 and 5, low expression) and category 2 (scores 6, 7 and 8, high expression). *CCL2* expression was scored by two independent researchers without knowing the identity or clinical outcome of patients.

Real-time quantitative RT-PCR

The *CCL2* RNA concentration in paraffin-embedded tissue sections was measured by real-time quantitative RT-PCR (qRT-PCR), using PALM Laser Micro-Beam System (PALM Microlaser Technologies AG, Bernried, Germany), dissected tissue material and the TaqMan technology.[30] Five μm paraffin sections of the cervical cancer tissues were cut and mounted on a glass slide coated with a 1.35 μm polyethylene layer PALM® LPC-Membrane (PALM Microlaser Technologies AG). After drying (overnight, 37°C), paraffin wax was removed and tissue sections were again dried (at least 4 h, 37°C). Cervical carcinoma tumor fields were selected and microdissected into 50 μl of lysis buffer [20 mM Tris (pH 7.4), 1 mM EDTA (pH 8.0), 2% sodium dodecyl sulphate].[31] 500 μg/ml proteinase K (Invitrogen Corporation, Carlsbad, Germany) was added and incubated for 16 h at 60°C. After centrifugation of the samples at 13 000 *g* for 5 min at 4°C, total RNA was extracted by adding 500 μl TRIzol® Reagent (Invitrogen Corporation) according to the manufacturer's protocol. After dissolving the RNA in 10 μl of RNase-free water, reverse-transcription was performed according to the manufacturer's protocol for 90 min at 42°C in a final volume of 20 μl containing 1x AMV-RT buffer (Roche Diagnostics, Mannheim, Germany) and random primers (Invitrogen Corporation). QRT-PCR was done on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Besides *CCL2*,[32] three housekeeping genes were also measured: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine guanine phospho-ribosyltransferase (*HGPRT*) and TATA box-binding protein (*TBP*)[33] (Table 1). Primers and probes were chosen to span introns, therefore avoiding the detection of DNA. For each measured gene, a

calibration curve was determined using a serial dilution of total RNA from healthy human spleen tissue. The average expression level of the housekeeping genes was calculated for each sample as well as the relative RNA concentration of *CCL2*, normalized to the housekeeping genes. The average of the samples belonging to the same RISH category was calculated and the category with high *CCL2* expression was set to 1.00.

Table 1. Primers and probes used for qRT-PCR

Gene	Primers and probes		Label	GenBank accession No
CCL2	forward	5'-tcgctcagccagatgcaat-3'		S71513
	reverse	5'-ccacaatggtcttgaagatcaca-3'		
	probe	5'-tcaccagcagcaagtgtcccaaagaa-3'	FAM	
HPRT	forward	5'-tgacactggcaaaacaatgca-3'		NM_000194
	reverse	5'-ggtccttttcaccagcaagct-3'		
	probe	5'-cttgaccatctttggattatactgcctgacca-3'	TET	
GAPDH	forward	5'-ttccaggagcgagatccct-3'		NM_002046
	reverse	5'-cacccatgacgaacatggg-3'		
	probe	5'-cccagccttctccatggtggtgaa-3'	FAM	
TBP	forward	5'-cacgaaccacggcactgatt-3'		X54993
	reverse	5'-ttttcttgctgccagtctggac-3'		
	probe	5'-tgtgcacaggagccaagagtgaaga-3'	FAM	

Preparation of cervical cancer cell suspensions for flow-cytometric analysis

The method for DNA flow-cytometric analysis of single cell suspensions from paraffin-embedded material, as described by Leers *et al*,[34] was optimized for cervical cancer. Two 50 μm thick sections were cut from five formalin-fixed, paraffin-embedded cervical cancer cases and placed in 10 ml glass tubes. The sections were dewaxed three times for 5 min in 3 ml of xylene and rehydrated in a descending ethanol range. After a wash in 3 ml of PBS, the sections were immersed in 3 ml of cold citrate solution (10 mM, pH 6.0) and incubated for 80 min at 80°C in a water bath. The sections were allowed to cool down to room temperature, centrifuged (5 min, 500 *g*), and digested for 10 min at 37°C in a water bath in 3 ml of 0.05 M Tris-HCl buffer complemented with 10 mM $CaCl_2$ and 0.1% trypsin (Sigma). Three milliliters of PBA was added to block the digestion, which was followed by mechanical treatment by mincing with a pipette tip. Next, the samples were filtered through a 50 μm mesh nylon filter and

washed three times in 3 ml of PBA (5 min, 500 *g*). The cell pellet was resuspended in PBA and the single cell suspension was aliquoted into samples of $1x10^6$ cells. To each sample, 100 μl of MAb directed against keratin [AE1/AE3 (1:100) and MNF116 (1:50; DAKO) diluted 1:40 in PBA] was added. After overnight incubation at room temperature, the samples were washed twice in PBA. Next, 100 μl of the secondary antibody fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG1 $F(ab')_2$ (DAKO) diluted 1:100 in PBA was added to the pellet. Incubation for 30 min in the dark was followed by washing three times with 3 ml of PBA. DNA staining solution was prepared in PBA, containing 0.1% ribonuclease A (RNase; Sigma) and 1 μM propidium iodide (PI; Calbiochem, San Diego, CA). Cells were incubated with 0.5 ml of DNA staining solution for 30 min at 37°C to activate the RNase. The samples were kept at 4°C for at least 2.5 h in the dark before flow-cytometric analysis and sorting. Negative control samples omitting the primary antibody were treated the same way.

Flow sorting

Flow sorting was performed using a FACStar Plus flow cytometer (BD Biosciences). A life gate was set on the FL3-W versus FL3-A pulse processor. Cells were sorted based on keratin expression (FITC) and DNA content (PI). Both positive and negative cell fractions were collected for DNA extraction.

DNA extraction

DNA from 11 flow-sorted tumor cell samples was extracted by the method described by Abeln *et al*.[35] In short, flow-sorted tumor cells and normal cells were resuspended in isolation buffer [0.3 mg/ml proteinase K in 10 mM Tris-HCL (pH 8.3), 1 mM EDTA, 0,5% Tween 20] at a concentration of 1000 cells/μl. After overnight incubation at 56°C in a water bath and 5 min inactivation at 100°C in a heat block, 1000 cells were used as a template for the PCR reactions.

Analysis of loss of heterozygosity

Two sequence tagged site (STS) markers (D17S1880 and D17S1872) were chosen to measure if the *CCL2* gene was lost. DNA was extracted from flow-sorted keratin-positive (cervical cancer cells) and keratin-negative (stromal and

inflammatory cells) material. Subsequently, this material was investigated for loss of heterozygosity (LOH) using the following fluorescein-labeled primers: D17S1880, 5'-GTTTCTTGACAGAATTTGAACACTTTG-3' and 5'-AGGGATTGCTTGAGCC-TET-3'; D17S1872, 5'-AATTGGGTCCAGAGAGCA-3' and 5'-CCAACTCTAGGACTGGGG-TET-3' (Isogen Bioscience BV, Maarssen, The Netherlands). The unlabeled primer of D17S1880 was modified (underlined part of sequence) to eliminate misinterpretation of the profile of the allele.[36] As a positive control, normal endometrial tissue of the same samples was investigated. Polymerase chain reaction (PCR) amplification was carried out in a 12 μl reaction buffer mixture, containing 2 mM $MgCl_2$, 0.01% BSA, 0.2 mM of each deoxynucleotide-triphosphate, 1.25 units of AmpliTaq Gold™ (Applied Biosystems, Foster City, CA), and 6 *p*mol of both oligonucleotide primers. PCR cycles were 5 min at 96°C, 33 cycles of 45 s at 94°C, 1 min 5 s at 55°C, and 45 s at 72°C followed by 6 min at 72°C in a GeneAmp 9700 thermocycler (Applied Biosystems). PCR products were run on an ABI PRISM™ 310 Genetic Analyzer for 20 min and analysed with GeneScan Analysis® 3.1 (both Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands).[37] The allelic imbalance factor was determined and the range for LOH was defined as LOH ≤0.55 or ≥1.80. The cut-off points were previously empirically determined by Cleton-Jansen *et al*.[38]

Statistical analysis

Data from immunohistochemistry as well as RISH are given as the mean ± the standard deviation (SD). Statistical analysis was performed using SPSS 10.0 (SPSS Inc, Chicago, IL). Data were processed using either the chi-square test or the Fisher's exact test, depending on the number and distribution of the compared groups. Kaplan-Meier survival curves were generated to assess differences in relapse-free period (defined as the observation time in months from surgery to relapse of the disease) or survival (defined as time in months from surgery to death due to cervical cancer). $P<0.05$ was considered statistically significant.

RESULTS

Cervical cancer cells express *CCL2*

In the present study, we have investigated the *CCL2* expression of cervical carcinoma cells, using a RISH technique, in 93 cervical cancer tissues. Both the percentage of positive tumor cells and the intensity of cytoplasmic staining were scored. As a control for the quality of the mRNA in this material, the expression of *β-actin* mRNA was measured in all the samples. All tissue samples strongly expressed *β-actin* mRNA. Inflammatory cells in the stroma that are known to produce *CCL2* (especially TAM) served as an internal positive control. Of the 93 tissue samples, 15 samples did not show *CCL2* staining (16%), 31 showed low *CCL2* expression (33%), and 47 high expression of *CCL2* (51%) (Figure 1). Five of the seven adenocarcinomas were positive for *CCL2* expression, whereas all six adenosquamous tumors were observed to express *CCL2*. To confirm the validity of our semi-quantitative scoring system for the RISH results, we also measured the relative expression of *CCL2* in 15 selected cervical carcinoma samples. Five tissue samples were randomly chosen from each *CCL2* expression category (five from category 0, five from category 1, and five from category 2). To minimize contamination with stromal or inflammatory cells, tumor fields were selected and isolated using the PALM Laser Micro-Beam System. The mean mRNA expression of *CCL2* using qRT-PCR with the TaqMan probe was for (RISH) category 0, 0.10 ± 0.06; category 1, 0.22 ± 0.07; and category 2, 1.00 ± 0.24. The significant correlation (P=0.005) between the RISH and QRT-PCR results demonstrates that the scoring system used for the RISH is indeed a semi-quantitative assessment of the amount of *CCL2* mRNA (Figure 2).

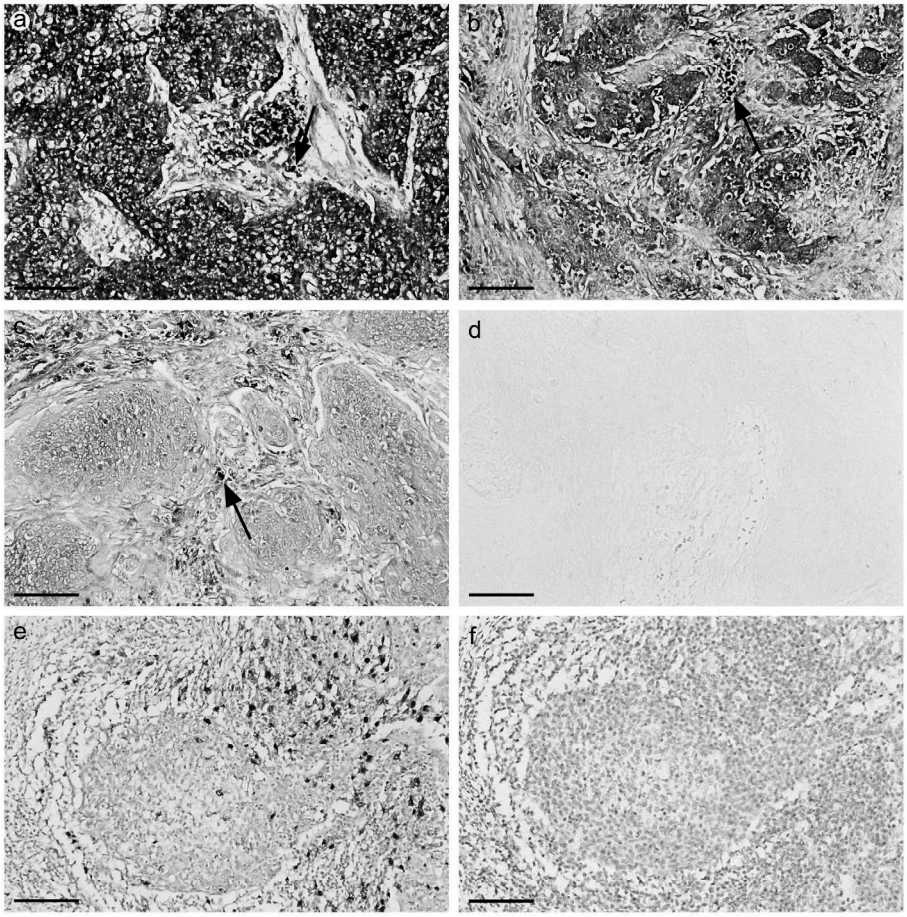


Figure 1. *CCL2* (RISH) and CD68 (immunohistochemical) staining for monocytes/macrophages in cervical carcinoma. Panels (**a**), (**b**) and (**c**) represent strong, weak and no expression of *CCL2* mRNA respectively. (**d**) Sense probe for *CCL2* (negative control). Monocytes/macrophages are indicated by arrows. Panels (**e**) and (**f**) represent positive and negative immunohistochemical staining of monocytes/macrophages (anti-CD68), respectively; sections are counterstained with Mayer's haematoxylin. (**a**-**f**) Scale bar = 100 μm. *See page 191 for color figure.*

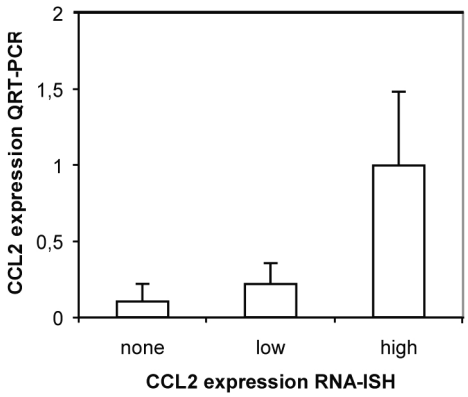


Figure 2. Correlation of *CCL2* mRNA expression identified by RISH and qRT-PCR. The results of *CCL2* RISH on paraffin-embedded cervical cancer tissue were compared with the results of a qRT-PCR using PALM Laser Micro-Beam System dissected paraffin-embedded cervical cancer tissue sections. Techniques are specified in the Materials and methods section.

Association between *CCL2* expression by cervical cancer cells and the number of TAM

CCL2 is known to attract human monocytes, memory T cells and NK cells.[13, 14] Therefore, the presence of *CCL2* mRNA in cervical carcinoma cells was associated with the amount of inflammatory infiltrate (comprising T cells, B cells, TAM, granulocytes, and NK cells).[39] The inflammatory infiltrate was quantified in three categories (minor, moderate, and extensive).[28] No significant association was found between *CCL2* expression (absent or present) and the amount of inflammatory infiltrate (P=0.982) (data not shown). Because CCL2 has been shown to be involved in the attraction of monocytes to the tumor site,[15-19] a relationship between *CCL2* expression by cervical carcinoma cells and the number of TAM was subsequently determined. TAM were predominantly located in the tumor stroma, although they were also observed between the tumor cells. The number of TAM in the tumor stroma showed a significant correlation with the number of TAM within the tumor fields (Figure 3; r^2=0.58, P<0.001). A positive correlation between *CCL2* score and the number of TAM in the tumor parenchyma, the tumor stroma, and the total number of TAM was observed (Figure 4; P<0.03, P=0.0008 and P=0.0017, respectively). Based on the prominent relationship between the number of stromal TAM and the *CCL2* score,

stromal TAM were divided into two groups (according to the median value of 20) and associated with the expression of *CCL2* by cervical carcinoma cells (none, low, high). The three groups differed statistically significantly from each other (*P*=0.001) and a shift from the group with <20 TAM per high-power field of view (HPF) to the group with ≥20 TAM per HPF was evident (Table 2).

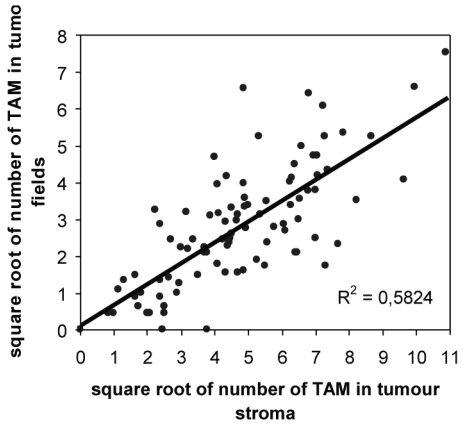


Figure 3. Correlation between the number of TAM within the tumor fields and in the tumor stroma. TAM were stained immunohistochemically with an anti-CD68 MAb as described in the Materials and methods section. The square roots of the number of TAM are displayed. The square roots are computed to make variances approximately constant.

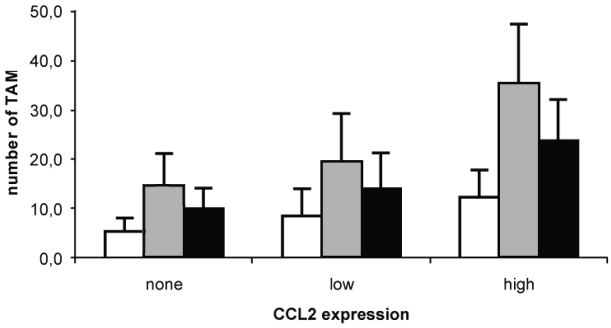


Figure 4. Relationship between *CCL2* mRNA expression (none, low, high) in cervical tumor cells and the number of TAM in the tumor parenchyma (open bars), the tumor stroma (grey bars), and the total number of TAM (black bars) in cervical cancer tissue sections. *CCL2* mRNA expression was detected by RISH and TAM were stained immunohistochemically with anti-CD68 MAb as described in the Materials and methods section.

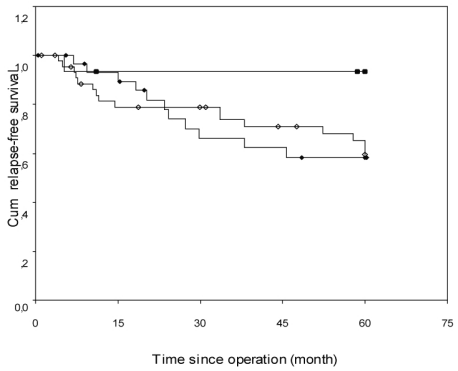
Table 2. Association between *CCL2* expression and low or high number of tumor-associated macrophages (TAM)

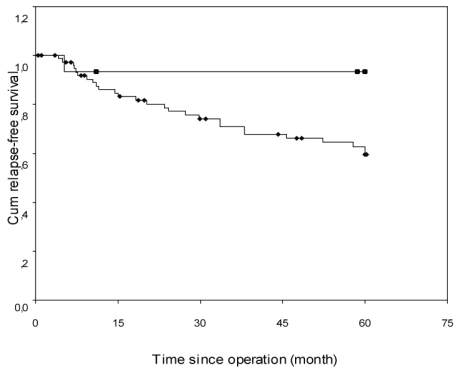
TAM	*CCL2* expression in cervical carcinoma cells			Significance *P*-value
	Absent	Low	High	
<20 per HPF	9*	22	14	**0.001**†
≥20 per HPF	6	9	33	

***** number of patients/cervical carcinomas
† chi-square test

Correlation between *CCL2* expression by cervical cancer cells and clinicopathological parameters

Subsequently, *CCL2* expression of cervical carcinoma cells was associated with clinicopathological parameters. No significant association was found with histology, HPV status, depth of infiltration or parametrial invasion. *CCL2* expression by cervical carcinoma cells was associated with post-operative radiotherapy, recurrent disease, tumor size, vascular invasion, lymph node metastasis and distant metastasis (Table 3). Considering these latter parameters, an increase in *P* value (*P*=0.010 compared with *P*=0.045 for *CCL2* expression) was found only in the case of tumor size and number of stromal TAM (≥20). A Kaplan-Meier plot was generated to determine the relationship between *CCL2* expression and cumulative disease-free survival (DFS) over a 5-year period. Since there was no significant difference between low and high *CCL2* expression (Figure 5A; log-rank test *P*=0.695) and DFS, these categories were combined leading to two categories (either with or without *CCL2* expression; log-rank test, *P*=0.030, Figure 5B). In addition, the cumulative overall survival was plotted (log-rank test, *P*=0.024, Figure 5C). The mean cumulative overall survival of the *CCL2*-negative category was 90 ± 21 months, which was statistically significantly different from the *CCL2*-positive category, which had an overall survival of 60 ± 24 months (*P*=0.024; spanning a maximal follow up of 10 years). In a multivariate analysis against two of the most predictive prognostic parameters, lymph node metastases and recurrence of disease, absence of *CCL2* was not statistically significant (data not shown).





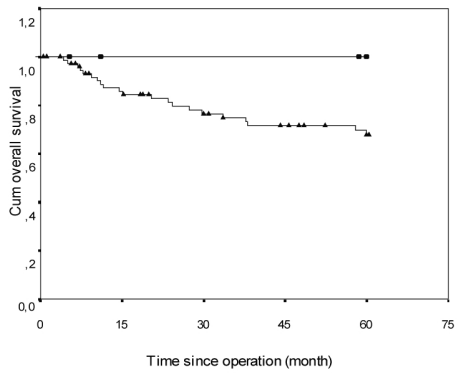


Figure 5. Cumulative relapse-free survival and cumulative overall survival stratified by *CCL2* mRNA expression in cervical carcinoma cells. Detection of *CCL2* mRNA and Kaplan-Meier analysis are specified in the Materials and methods section. (**A**) Cumulative disease-free survival stratified by *CCL2* mRNA expression [none (circle), low (diamond) and high (triangle)] in cervical carcinoma cells. (**B**) Cumulative disease-free survival stratified by *CCL2* mRNA expression [absent (circle) and present (low and high) (triangle)] in cervical carcinoma cells. (**C**) Cumulative overall survival stratified by *CCL2* mRNA expression [absent (circle) and present (low and high; triangle)] in cervical carcinoma cells.

Table 3. Summary of clinicopathological features of the patients and tumors.

Patient and tumor characteristics		*CCL2* expression in cervical carcinoma cells		Significance *P*-value
		Negative	Positive	
Post-operative RT	No	11	34	**0.048**
	Yes	4	44	
Recurrent disease (within 5 years)*	No	14	50	**0.023**
	Yes	1	27	
Histology	Squamous	13	59	0.507
	Adeno and Adenosquamous	2	19	
HPV status*	16, 18	10	57	0.197
	Other	0	14	
Tumor size*	<40 mm	12	49	**0.045**
	≥40 mm	1	26	
Depth of infiltration*	<15 mm	11	49	0.502
	≥15 mm	4	22	
Parametrial invasion*	No	15	62	0.054
	Yes	0	15	
Vascular invasion*	No	10	30	**0.049**[†]
	Yes	5	46	
Lymph node metastasis	No	14	54	**0.045**
	Yes	1	24	
Distant metastasis*	No	15	55	**0.011**
	Yes	0	22	

Statistical analysis was based on Fisher's exact test.
* Only data for cervical carcinoma samples with a determined HPV type were included; other types included HPV31 (*n*=2), HPV33 (*n*=6), HPV35 (*n*=1), HPV45 (*n*=2), HPV58 (*n*=1), HPV59 (*n*=1), HPV68 (*n*=1).
[†] chi-square test.

Absence of *CCL2* expression is associated with genetic alterations near the *CCL2* (17q11.2) genomic region

To determine if the absence of RISH staining was due to genetic alterations near the *CCL2* (17q11.2) genomic region, LOH analysis of the *CCL2* gene was performed using six tissue samples without *CCL2* mRNA expression and five with *CCL2* mRNA expression. The tissue samples were randomly selected from both groups. Two STS markers, D17S1880 positioned at the centromeric side and D17S1872 positioned at the telomeric side of the *CCL2* gene, were chosen. To obtain pure tumor cell populations and to avoid contamination with stromal or inflammatory cells, dissected

tumor cells were sorted on the basis of their keratin expression by flow cytometry. LOH was found in five of the six samples without *CCL2* expression (Figure 6). Only one tissue sample, as well as the five control samples with high *CCL2* expression, showed retention. Comparison of both groups (with and without *CCL2* expression) showed a statistically significant difference (*P*=0.015).

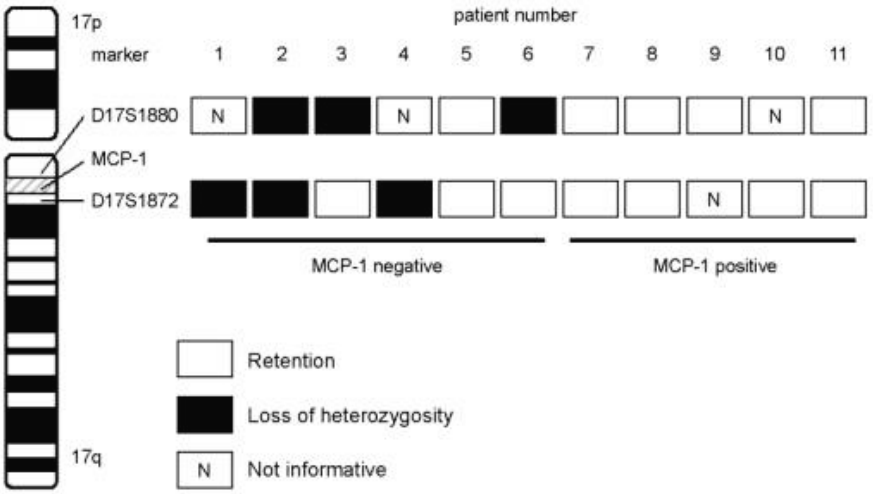


Figure 6. LOH of the *CCL2* (*MCP-1*) genomic region in cervical carcinoma, assessed using STS markers D17S1880 and D17S1872. Five tumors without *CCL2* mRNA expression displayed LOH at either one side or both sides of the *CCL2* gene. In five tumors (7-11) with *CCL2* expression, no LOH was found. The LOH technique is specified in the Materials and methods section.

DISCUSSION

In the present study we found a relationship between *CCL2* expression by cervical tumor cells, the presence of TAM, and clinicopathological parameters. In our study, 84% of the cervical cancers showed staining for *CCL2* mRNA. In an earlier study with a limited number of tumor samples, Riethdorf *et al*[16] found a comparable number of squamous cell carcinomas of the cervix (three of five, 60%) to be positive for CCL2. In a more recent study by Kleine-Lowinsky *et al*,[40] no expression of *CCL2* was found in four cases of cervical carcinoma. The reason for the discrepancy between our study, Riethdorf *et al*'s study, and the latter study is not clear, but it might be related to the

smaller number of tumor samples used in the study by Kleine-Lowinsky *et al*. Cervical cancer cells have the capacity to produce CCL2, as demonstrated by the constitutive CCL2 production of HeLa and inducible CCL2 expression of SiHa.[41] The expression of CCL2 by cervical cancer cells *in vivo* may be either repressed by expression of E6 or E7 proteins[41] or induced by stimuli (e.g., cytokines) from the tumor environment that induce nuclear factor (NF)-kappaB activation.[42] Also, in other tumor types, such as breast cancer, pancreatic, gastric and esophageal cancer, a variable proportion of the tumor cells were found to express CCL2.[15, 17, 24, 25, 43, 44]

Using RISH, we have demonstrated that the majority of cervical carcinoma cells from tumors classified as FIGO stage IB or IIA express *CCL2* mRNA. The semi-quantitative scoring method used to quantify the results[29] was substantiated using qRT-PCR. The observation that category 0 [no *CCL2* expression detectable (RISH)] did show *CCL2* expression with the qRT-PCR technique indicates that the latter method is more sensitive. Similar findings with regard to the increased sensitivity of the qRT-PCR technique compared with RISH have been described by other groups.[45, 46]

As previously shown, CCL2 has been identified as a major chemokine inducing the recruitment of macrophages to human tumors such as those of the cervix,[16] ovary,[47, 48] lung,[18, 49] and breast.[17, 44] Also, in our study, *CCL2* mRNA expression was associated with the number of TAM. This association was observed only for TAM and not for the extent of inflammatory infiltrate present. The lack of a significant correlation between *CCL2* expression and the amount of inflammatory infiltrate, the significant association between tumor-derived *CCL2* and TAM, and the association between *CCL2* expression and survival support the concept that TAM play a role in tumor progression of cervical carcinoma. This view is strengthened by the observation that high numbers of stromal TAM (>20) are associated with tumor size. This view that inflammatory tumor-educated macrophages promote tumor progression and metastases is becoming more and more accepted.[12, 20-22] It is thought that the inflammatory infiltrate is attracted to the tumor site and remains at the tumor site through the action of cytokines such as macrophage colony-stimulating factor (M-CSF) and CCL2. However, as observed in a previous study, the number of TAM does not correlate significantly with relapse in cervical cancer.[50] This may be explained by the complexity of signals provided by the multitude of cytokines and chemokines

present in the tumor environment, and the presence of different subpopulations of TAM that may respond differently to these signals.

Cervical cancer patients without *CCL2* expression had the most favorable prognosis. Similar results were reported by Ueno *et al*[17] in a group of breast cancer patients. It should be noted that in the latter study, the CCL2 production of tumors (thus including stroma, TAM, and cancer cells) was associated with survival, whereas in our study, *CCL2* mRNA expression by cancer cells exclusively was associated with survival. Although there is a positive relationship between TAM and *CCL2*, only the absence of *CCL2* is significantly associated with prognosis. It is noteworthy that the only patient who died from disease in the group without detectable *CCL2* was a patient with a high number of TAM (over 40 stromal TAM per HPF). This high number of TAM may have been induced by the presence of other cytokines such as M-CSF. In the group of patients with high expression of *CCL2*, it was observed that high expression was associated with a high number of TAM. However, even if *CCL2* was expressed by the tumor, the number of TAM was low (less than 20), suggesting that other factors also play a role in the recruitment and survival of TAM.[22, 51]

Patients divided into groups with or without postoperative pelvic radiotherapy[52] differed in *CCL2* expression. The statistically significant association of *CCL2*-positive cervical cancer cells with relapse and post-operative radiotherapy suggests that *CCL2*-expressing tumor cells behave in a more malignant fashion. This behavior is supported by the observations of Neumark *et al* who reported that murine mammary adenocarcinoma cancer cell clones with a high malignant phenotype expressed high levels of CCL2 in contrast to their low malignant phenotype counterparts.[53]

Lack of expression of *CCL2* RNA correlated with LOH in the region containing the *CCL2* gene (chromosome 17q11.2). Five of the six samples without detectable *CCL2* expression investigated showed LOH of one or both STS markers flanking the *CCL2* gene, suggesting that LOH is a mechanism by which *CCL2* RNA expression is prevented. However, loss of one *CCL2* allele does not explain the lack of *CCL2* RNA expression. The absence of *CCL2* RNA expression suggests that the other *CCL2* allele is also impaired (e.g., a mutation in the *CCL2* gene or methylation of the *CCL2* promoter). Similar results were found for the relationship between absence of mRNA expression of the metastasis suppressor gene *KiSS1* and LOH on chromosome 6q16.3-q23 in the progression of melanoma.[54]

Our data from cervical carcinoma uphold the concept that tumors modify their environment by expressing particular cytokines and chemokines, thus modifying the function of TAM in such a way that tumor progression rather than an anti-tumor response is supported.

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CHAPTER 3

Role of tumor derived pro-inflammatory cytokines GM-CSF, TNF-α and IL-12 in the migration and differentiation of antigen-presenting cells in cervical carcinoma

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ABSTRACT

Pro-inflammatory cytokines are important in modifying the activity, differentiation, and migration of antigen-presenting cells (APC) and may influence the survival of cancer patients. The study assessed whether granulocyte-monocyte colony-stimulating factor (*GM-CSF*), tumor necrosis factor (*TNF*)-α and interleukin (IL)-12, expressed by cervical cancer cells, are important for the activity, differentiation, and migration of APC.

In 90 patients with cervical carcinoma the number of monocytes/tumor associated macrophages (TAM), mature dendritic cells (DC), and Langerhans' cells (LHC) was determined using immunohistochemistry. An RNA-*in situ* hybridization technique was used to measure the expression level of *GM-CSF*, *TNF-α*, *IL-12p35* and *IL-12p40*. Tumor-associated macrophages were detected intraepithelial as well as in the stroma of the tumor. LHC were only detected intraepithelial and mature DC only in the tumor stroma. The number of TAM correlated positively with the number of mature DC. The expression levels of *GM-CSF* and *TNF-α* correlated positively with the number of TAM and DC. *TNF-α* showed a negative correlation with the number of LHC. A significant correlation between the expression of functional IL-12 (*IL-12p40*) and stromal TAM was found. The expression of *GM-CSF*, *TNF-α* and *IL-12p40* did not correlate significantly with disease-free survival. However, high *IL-12p40* expression was associated with a favorable cumulative overall survival. These results suggest that *GM-CSF* as well as *TNF-α*, produced by cervical carcinoma cells, may play a role in the differentiation of monocytes into mature DC. Furthermore, *TNF-α* may influence the migration of LHC from the tumor.

INTRODUCTION

Cervical cancer is a leading cause of morbidity and mortality among women worldwide, especially in the developing countries.[1, 2] Infection with oncogenic types of human papillomavirus (HPV) is an important factor in the development of cervical cancer.[3, 4] The persistent HPV infection induces an inflammatory response. Cervical cancer cells are often surrounded by a large number of inflammatory cells. Approximately half of these cells are cells with antigen-presenting characteristics.[5] Although these cells are capable of generating an antitumor response, this does not occur.[6, 7] At present, it is thought that the inflammatory cells are suppressed in their antitumor properties and support tumor growth rather than eliminate cancer cells.[8, 9] Inflammatory cells are attracted to the tumor site by locally produced cytokines and chemokines.[10, 11] Cervical cancer cells are known to produce an extensive range of cytokines.[12, 13] In addition to attracting inflammatory cells, these cytokines influence the activation status and function of the infiltrating antigen-presenting cells (APC) and the stroma and thus influence the course of the disease.[11, 14] We have shown that expression of chemokine (C-C motif ligand 2 (*CCL2)* by the cervical cancer cells is associated with an increased number of tumor-associated macrophages (TAM) and a decreased disease-free survival of cervical carcinoma patients.[15] Furthermore, we have observed a down regulation of the expression level of pro-inflammatory cytokines, such as granulocyte-monocyte colony-stimulating factor (*GM-CSF*), tumor necrosis factor (*TNF*)-*α* and interleukin (*IL*)-*12* in cervical carcinoma cell lines.[12] GM-CSF, TNF-*α* and IL-12 are important in profiling immune reactivity.[10, 16] GM-CSF and TNF-*α* are involved in differentiation and survival of dendritic cells (DC) and Langerhans' cells (LHC) and IL-12 is known to stimulate effector cell populations such as cytotoxic T cells and natural killer (NK) cells.[17, 18] Our observations *in vitro* are consistent with a hypothesis that presumes that increased local proinflammatory cytokine expression would promote antitumor immunity. This suggests that patients with cervical cancer producing these proinflammatory cytokines may follow a relatively more benign course.

To test this hypothesis we quantified different subsets of APC (TAM, LHC and DC) and measured the mRNA expression levels of *GM-CSF*, *TNF-α* and *IL-12* in tumors of 90 patients with cervical carcinoma. Subsequently, we determined the association

between mRNA expression with clinicopathological parameters, disease-free survival (DFS), and cumulative overall survival (OS) of the cervical carcinoma patients.

MATERIALS AND METHODS

Patient material

Between 1985 and 1995, 254 untreated patients suffering from primary cervical carcinoma with stage IB and IIA underwent a radical hysterectomy type III with lymphadenectomy. From the tissue obtained, 90 tissue samples were available for research. Tissues were routinely embedded in paraffin after 10% formalin fixation. The tissue samples of each patient were examined by a pathologist for the presence of tumor. Tumor percentage varied between 30% and 80%. The characteristics of the patients are depicted in Table 1. Human tissue samples were used according to the guidelines of the Ethical Committee of the Leiden University Medical Center.

Table 1. Summary of clinicopathologic features of patients and tumors

Patient and tumor characteristics	Outcome	N*
Age	46, mean	90
	29 – 76, range	
FIGO	IB	68
	IIA	22
Lymph node metastasis	No	66
	Yes	24
Tumor size†	< 40 mm	67
	≥ 40 mm	18
Depth of infiltration†	< 15 mm	61
	≥ 15 mm	23
Vascular space involvement†	No	39
	Yes	49
Parametrial invasion†	No	75
	Yes	14
HPV status‡	16, 18	65
	Other	14
Histology	Squamous	77
	Adenosquamous	6
	Adeno	7

HPV indicates human papillomavirus.

* N, number of patients/cervical carcinomas.

† The number of reported cases is affected by incidental missing cases.

‡ Only data for cervical carcinoma samples with a determined HPV type were included, other subtypes included HPV31 (*n*=2), HPV33 (*n*=6), HPV35 (*n*=1), HPV45 (*n*=2), HPV58 (*n*=1), HPV59 (*n*=1), HPV68 (*n*=1).

Preparation of GM-CSF-, TNF-α-, IL-12p35- and IL-12p40-probe

RNA was isolated from frozen human spleen using TRIzol® (Invitrogen, Breda, The Netherlands) and first-strand cDNA synthesis with oligoDT primers and reverse transcriptase AMV (both Roche Diagnostics, Mannheim, Germany) were both done according to the manufacturer's instructions. Oligonucleotide primers for *GM-CSF*, *TNF-α*, *IL-12p35* and *IL-12p40* were chosen on the basis of known sequences (see Table 2) and cDNA encoding for the different cytokines was amplified. A pGEM®-3Zf(+) Vector (Promega, Madison, WI) was linearized with SmaI and the polymerase chain reaction (PCR) products were cloned into the vector. After transferring the vector to *E. coli* strain Top 10 (Invitrogen Corp., San Diego, CA) the plasmids were isolated by using the QIAfilter Maxi KITS protocol (QIAGEN, Hilden, Germany). The sequence of the PCR product was confirmed by DNA sequencing. Plasmids were linearized with BamH1 and EcoR1 (both Boehringer, Mannheim, Germany) in the case of *GM-CSF*, *TNF-α* and *IL-12p40* or BamH1 and SacI in the case of *IL-12p35* using One-Phor-All Buffer Plus (Amersham Biosciences, Roosendaal, The Netherlands). Both strands were translated in a digoxigenin (DIG)-labeled RNA probe according to manufacturer's instructions (Roche Diagnostics). The concentration of the DIG-labeled sense and antisense RNA probes were determined on a 1% agarose gel stained with ethidium bromide (Sigma, St. Louis, MO). Probes were stored at -20°C until further use.

RNA-*in situ* hybridization

The RNA-*in situ* hybridization (RISH) was performed as previously described.[19, 20] In brief: 3 *μ*m thick paraffin sections were pretreated and hybridized with 100 ng/ml DIG-labeled RNA probe diluted in hybridization mixture containing NaCl and saline-sodium citrate (SSC; Table 2). Hybridization was allowed for 16 h at either 50°C (*GM-CSF* and *TNF-α*) or 42°C (*IL-12p35* and *IL-12p40*) in a humidified chamber. Slides were washed for 30 min in 2x SSC followed by 45 min in 0.1x SSC with 20 mM *β*-mercaptoethanol (Merck, Darmstadt, Germany), both at the hybridization temperature (see Table 2). Subsequently, the slides were incubated for 30 min with 2 U/ml ribonuclease (RNase) T1 (Roche Diagnostics) in 2x SSC, 1 mM EDTA at 37°C. RNA hybrids were detected using mouse anti-DIG (1:2000, Sigma-Aldrich Chemie, Steinham, Germany), rabbit anti-mouse Ig (1:50, DAKO, Glostrup, Denmark), and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP, DAKO).[12]

Table 2. RNA probes used and RNA-*in situ* hybridization conditions

Target	Primer	Sequence (5'-3')	Product (bp*)	Accession number	Position	Hybridization temperature and SSC concentration
GM-CSF	forward reverse	TCAGAAATGTTTGACCTCCAGG TCCCATTCTTCTGCCATGC	430	NM_000758	189-618	50°C, 1x SSC†
TNF-α	forward reverse	AGATCATCTTCTCGAACCCCG AAGGTTGGATGTTCGTCCTCC	492	X01394	384-875	50°C, 2x SSC
IL-12p35	forward reverse	TGCTCCAGAAGGCCAGACAAAC CCCGAATTCTGAAAGCATGAAG	465	XM_003121	320-784	42°C, 2x SSC
IL-12p40	forward reverse	GGACCAGAGCAGTGAGGTCTT CTCCTTGTTGTCCCCTCTGA	373	XM_004011	189-561	42°C, 1x SSC

*bp indicates basepair; †SSC indicates saline-sodium citrate.

Immunohistochemistry

Serial sections, 3 μm thick, of formalin-fixed and paraffin-embedded tissue were mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by treating the sections either for 10 min at 37°C using 1 mM EDTA pH 8.0 (mouse anti-CD1a) or by treating the sections for 10 min at 37°C with 0.1% trypsin (Sigma-Aldrich Chemie), 0.1% $CaCl_2$, pH 7.4 (mouse anti-CD68 and mouse anti-DC-Lamp). Sections were incubated with PBA [1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)], followed by an overnight incubation at room temperature with monoclonal antibody (MAb) directed against CD1a (1:100, IgG1 kappa, clone 1CA04; Neomarkers, Fremont, CA), CD68 (1:1600, IgG1, DAKO) or DC-Lamp (1:20, IgG1, clone 104.G4; Immunotech, Marseille, France). Subsequently, immunohistochemical staining was performed as previously described.[20] For all three tested antibodies human tonsil served as a positive control.

(Semi-)quantitative evaluation of RISH and immunohistochemistry

RISH was scored as previously described.[21] Intensity was scored as none (0), mild (1), moderate (2), or intense (3) at low magnification (x100). Furthermore, the percentage of positive tumor cells was determined and divided in groups (from 0 to 5): 0% (0, absent), 1% to 5% (1, sporadic), 6% to 25% (2, local), 26% to 50% (3, occasional), 51% to 75% (4, majority), and 76% to 100% (5, large majority). The sum of both the percentage and the staining intensity of the positive cells resulted in an overall score (0 or 2-8). The scores were combined into three groups: category 0 (score 0, no expression), category 1 (scores 2, 3, 4 and 5, low expression), and category 2 (scores 6, 7 and 8, high expression). mRNA expression was scored by two independent researchers without knowing the identity and clinical outcome of patients. CD1a-, CD68- and DC-Lamp-positive cells were quantified in the tumor by counting the number of stained cells per 6, randomly selected, high-power field of view (HPF, x400; Figure 1).

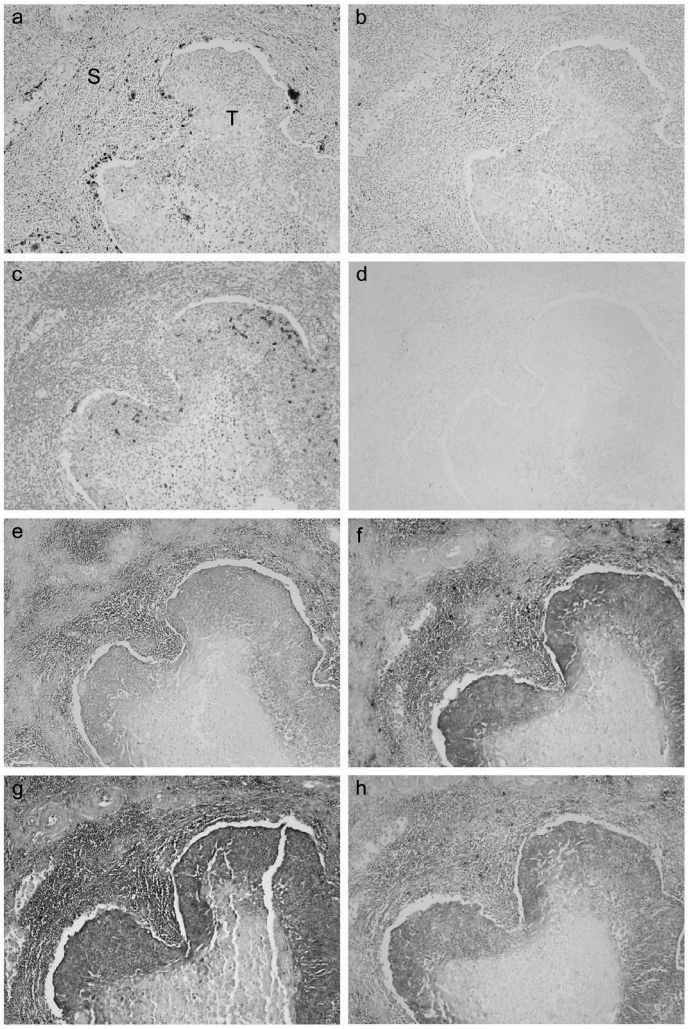

Figure 1. Immunohistochemical and RISH staining of paraffin sections of cervical carcinoma are shown. Carcinoma cells are indicated by a T, whereas S represents tumor stroma. (**a**-**c**) Immunohistochemical staining of cervical carcinoma. (**a**) Staining for monocytes and macrophages (anti-CD68). Staining is present intraepithelial as well as in the tumor stroma. (**b**) Staining for mature dendritic cells [DC (anti-DC-Lamp)] seen as nests in the tumor stroma. (**c**) Staining for Langerhans' cells [LHC (anti-CD1a)]. Staining can be seen intraepithelial. Positive cells are clearly visible by a dark-brown precipitation. Sections (**a**-**c**) are counterstained with Mayer haematoxylin. (**d**-**h**) RNA-*in situ* hybridization (RISH) for cytokines in the same cervical carcinoma sample as shown in (**a**-**c**). (**d**) Negative control. (**e**) *GM-CSF* shows a weak expression in both cervical carcinoma cells and in inflammatory cells in the tumor stroma. (**f**) *TNF-α* is strongly expressed in cervical carcinoma cells as well as in some of the cells in the tumor stroma. (**g**) *IL-12p35* is strongly expressed in the cervical carcinoma cells as well as in inflammatory cells in the tumor stroma. (**h**) *IL-12p40* shows a moderate expression in the tumor cells and a strong expression in some of the cells in the tumor stroma. Positive signal in RISH is seen as a (dark) blue precipitation. Sections (**d**-**h**) are not counterstained. Original magnification, x80. *See page 192 for color figure.*

Statistical analysis

Data from immunohistochemistry as well as RISH were given as the mean ± the standard deviation (SD). Statistical analysis was done using SPSS 12.0 (SPSS, Chicago, IL). Data were processed by using the chi-square test, the Kruskal-Wallis H test, and analysis of variance (ANOVA), depending on the number and distribution of the compared groups. Kaplan-Meier survival curves were generated to assess differences in disease-free period [defined as the observation time in months from surgery to recurrence of the disease (disease-free survival) (DFS)] or cumulative overall survival (defined as time in months from surgery to death due to cervical cancer) (OS). A Cox regression was used for multivariate survival analysis. $P<0.05$ was considered statistically significant.

RESULTS

Number and distribution of TAM, LHC and DC

To investigate the association between the number of APC, expression of proinflammatory cytokines by cervical cancer cells, and survival, the number of TAM, LHC and mature DC in the tumor was determined. TAM were observed most abundantly in the tumor stroma but were also present between the tumor cells (intraepithelial). LHC were observed only intraepithelial in similar numbers as TAM. In contrast to LHC, mature DC were only detected in the tumor stroma (Figure 1). The number of mature DC was lower than the number of TAM in the tumor stroma. There was a positive correlation between the number of stromal TAM and the number of DC (Figure 2; $r^2=0.371$, $P<0.001$). There was no significant association between either TAM and LHC or DC and LHC (data not shown).

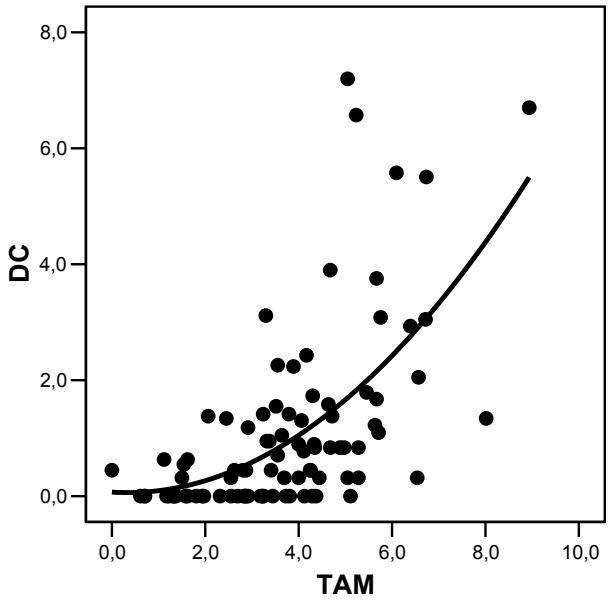

Figure 2. Graph shows association between the number of stromal tumor-associated macrophages (TAM) and mature dendritic cells (DC). A positive correlation between the number of stromal TAM compared with the number of mature DC was observed (quadratic regression, r^2=0.371, P<0.001). Displayed are the square roots of the counted cells.

Expression of *TNF*-α, *GM-CSF* and IL-12 (*IL-12p35* and *IL-12p40*) in cervical cancers

Subsequently, the expression of *TNF*-α, *GM-CSF,* and *IL-12* mRNA in cervical cancer was determined. Cancers were scored for staining intensity as well as percentage of cancer cells positive for cytokine expression. RNA of all the investigated cytokines could be detected, although in different expression patterns (Table 3). A positive correlation between *GM-CSF* and *TNF*-α expression was observed (data not shown, r^2=0.515, P<0.001).

Functional IL-12 is composed of 2 subunits (IL-12p35 and IL-12p40). Therefore, we measured both *IL-12p35* and *IL-12p40*. A positive correlation between *IL-12p35* and *IL-12p40* was found (data not shown, r^2=0.405, P<0.001). *IL-12p35* was more abundantly expressed than *IL-12p40*. All samples that expressed *IL-12p40* also expressed *IL-12p35*, indicating that *IL-12p40* is a parameter for functional IL-12.

Table 3. Correlation between *GM-CSF*-, *TNF-α*- and *IL-12p40* expression and the mean number of stromal TAM, mature DC and LHC in cervical carcinoma

				TAM			
Cytokine	N* (%)	Expression level		Stromal	Intraepithelial	DC	LHC
GM-CSF	45 (50)	Absent	(category 0)	12	8	2	9
	15 (17)	Low	(category 1)	21	13	7	6
	30 (33)	High	(category 2)	21	13	5	6
P-value				**< 0.001**	**< 0.001**	**< 0.001**	0.159
TNF-α	43 (48)	Absent	(category 0)	13	8	3	8
	31 (34)	Low	(category 1)	19	11	5	9
	16 (18)	High	(category 2)	23	14	3	3
P-value				**0.004**	**0.007**	**0.004**	**0.003**
IL-12p40	41 (46)	Absent	(category 0)	14	9	4	9
	28 (31)	Low	(category 1)	19	11	4	7
	21 (23)	High	(category 2)	18	11	2	5
P-value				**0.044**	0.115	0.172	0.252

TAM indicates tumor-associated macrophages; DC dendritic cells; LHC Langerhans' cells.
* N = number of tumors, total number was 90 tumors. The scores were combined into three groups: category 0 (score 0; absent expression), category 1 (scores 2, 3, 4 and 5; low expression) and category 2 (scores 6, 7 and 8; high expression) as described in Materials and methods section.
Statistical significant *P*-values are bold.

Association between *TNF-α*, *GM-CSF* and *IL-12* and APC in cervical cancer

Since TNF-α is known to participate in the attraction of inflammatory cells to the tumor site, we determined the association between expression of *TNF-α* and the number of stromal TAM.[8] Expression of *TNF-α* showed a positive correlation with the number of stromal TAM (Table 3; χ^2=11.084, *P*=0.004). As expected from the positive correlation between the expression of *TNF-α* and *GM-CSF*, also a positive correlation between *GM-CSF* and the number of TAM was found (χ^2=37.606, *P*<0.001).

TNF-α, as well as GM-CSF, are known to differentiate monocytes/macrophages (e.g., TAM) into mature DC.[22, 23] Indeed, an association between expression of *TNF-α* and the number of mature DC was found. Low expression of *TNF-α* was associated with more DC per HPF compared with the absence of detectable *TNF-α* expression (Table 3; χ^2=10.892, *P*=0.004). A similar correlation was found between *GM-CSF* and DC (χ^2=17.961, *P*<0.001). No further increase in the number of mature DC was observed in tumors displaying a high *TNF-α* or high *GM-CSF* expression level. In addition, an association between the number of intra-epithelial TAM and *TNF-α* and *GM-CSF* was found (Table 3; χ^2=9.952, *P*=0.007 and χ^2=15.790, *P*<0.001, respectively).

Expression of *TNF-α* showed a negative correlation with the number of LHC (Table 3; χ^2=11.478, *P*=0.003). No significant association was found between the expression level of *GM-CSF* and LHC.

A significant correlation between the expression of functional *IL-12* (*IL-12p40*) and stromal TAM was found (Table 3; χ^2=6.228, *P*=0.044). No significant association between intraepithelial TAM, mature DC and LHC and the expression level of functional *IL-12* was found.

Patients

Of the total group of 90 patients, 68 patients were diagnosed as FIGO stage IB and 22 as FIGO stage IIA and all underwent a radical hysterectomy combined with pelvic lymphadenectomy (Table 1). Twenty four of these patients had lymph node metastasis. Forty-seven patients received postoperative radiotherapy because of either tumor-positive lymph nodes or a combination of 2 of the following unfavorable prognostic parameters: depth of infiltration ≥ 15 mm or > 2/3 of the cervix, tumor size ≥ 40 mm and the presence of vasoinvasion. Twenty-seven patients suffered recurrent disease. Sixty-three patients were alive, 6 suffered from a recurrence, and 22 patients had died of disease; 5 died from causes unrelated to the primary disease, but showed no evidence of disease as concluded from the clinical record. FIGO stage and tumor size were associated with the presence (category 0 versus category 1+2) of *GM-CSF* (*P*=0.014 and *P*=0.022, respectively). No significant association with FIGO stage or tumor size with either the presence or expression level (low, category 0+1, or high, category 2) of *TNF-α, IL-12p35* or *IL-12p40* was found.

***TNF-α, GM-CSF, IL-12* expression and patient survival**

To investigate the relation between the expression of the investigated cytokines and survival, Kaplan-Meier plots were created. A log-rank test was used to determine statistical differences in survival. Neither *GM-CSF* expression, *TNF-α* nor *IL-12p40* expression showed a difference in disease-free survival (data not shown). However, there was a statistical difference in cumulative overall survival when comparing low expression of *IL-12p40* with no or high expression of *IL-12p40* (Figure 3; log-rank test 5.79, *P*=0.016 and log-rank test 4.59, *P*=0.032 respectively; Table 4). When performing a multivariate analysis using a Cox regression model, *IL-12p40* was not an

independent prognostic parameter in cervical carcinoma when tested against lymph node metastasis and depth of tumor infiltration (data not shown).

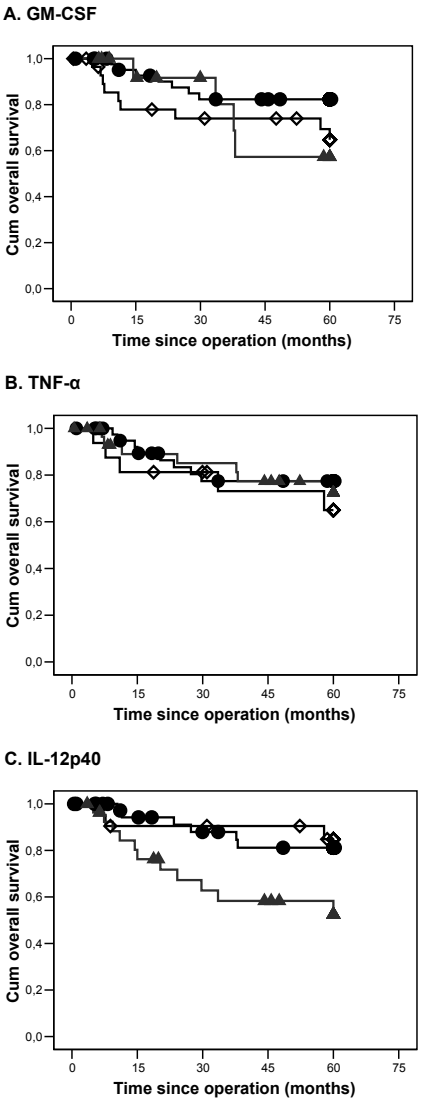

Figure 3. Graph shows association between *GM-CSF*, *TNF*-α, *IL-12p40* expression and cumulative overall survival in cervical carcinoma. No expression is represented as the curve with a circle; low expression level by a triangle; and high expression level by a diamond. Patients with low *IL-12p40* expression showed a worse prognosis compared with no or high *IL-12p40* expression (log-rank test 5.79, *P*=0.016 and log-rank test 4.59, *P*=0.032, respectively; Table 4).

Table 4. Association between *GM-CSF*, *TNF*-α, *IL-12p40* expression and cumulative overall survival in cervical carcinoma

	Compared expression levels		
Cytokine	No vs Low	No vs High	Low vs High
GM-CSF	1.60 (0.206)	2.67 (0.102)	0.00 (0.977)
TNF-α	0.11 (0.743)	0.68 (0.411)	0.31 (0.577)
IL-12p40	5.79 (**0.016**)	0.07 (0.793)	4.59 (**0.032**)

Shown are the log-rank test and (*P*-value) of compared expression levels.
Statistical significant *P*-values are bold.

DISCUSSION

In the present study we found a positive relationship between TAM and DC associated with *TNF-α*- and *GM-CSF*- expression and a negative relation between LHC and *TNF-α* expression in cervical carcinoma. There was no significant relation between *IL-12* expression and the investigated APC except for stromal TAM, but *IL-12p40* did show a correlation with cumulative overall survival. However, *IL-12p40* was not an independent prognostic parameter in cervical carcinoma when tested against lymph node metastasis and depth of tumor infiltration.

From the APC investigated, TAM were the most abundant. There was a significant correlation between the number of intraepithelial TAM and the number of TAM in the tumor stroma, suggesting that TAM migrate from the stroma to the parenchyma (data not shown). Although TAM have the capability to destroy tumor cells, at present it is thought that TAM support the progression of the tumor.[8, 9, 24] This is also supported by the study of Bingle *et al* who demonstrated that in the majority of the investigated cases the presence of an inflammatory infiltrate was detrimental to the patients.[25] Mature DC (DC-Lamp-positive cells) were only observed in the tumor stroma. The number of mature DC correlated with the number of TAM in the stroma, suggesting that a part of the total number of TAM in the stroma differentiates into mature DC. This is further supported by the correlation between *GM-CSF*, *TNF-α* and the number of mature DC. Both GM-CSF and TNF-*α* are known to play a role in the differentiation of monocytes into mature myeloid DC *in vitro*.[17] In contrast, the number of LHC did not correlate with either the number of TAM, or the number of

mature DC. It has been shown that in the presence of GM-CSF, TNF-*α* and TGF-*β*, DC may further differentiate into LHC.[17] Although in a previous report we have shown that *TGF-β* is expressed by cervical tumor cells, no significant association between either TAM and LHC or mature DC and LHC was observed, suggesting that no substantial differentiation from mature DC into LHC occurred.[26] This implies that the LHC are remnants of a resident population initially present in the epithelial layer of the cervix.[27]

Monocytes are known to be attracted to the tumorsite by certain chemokines.[28] We have previously shown that *CCL2* expressed by cervical tumor cells is associated with the presence of TAM at the tumor site.[15] In addition to *CCL2*, TNF-*α* expression is often correlated with large numbers of inflammatory cells.[8,29] Indeed, a positive association between *TNF-α* expression and stromal TAM was found. In the present study even a more pronounced association between the expression of *GM-CSF* and the number of TAM (both intraepithelial as well as in the tumor stroma) was found. This is in agreement with the previously reported effect of GM-CSF on migration of LHC/DC into organotypic cultures of HPV-transformed keratinocytes.[30] A similar positive correlation between the presence of GM-CSF, TNF-α and the number of mature DC in breast cancer tissue was reported by Tsuge *et al*.[31] In contrast, Trutmann *et al* did not report a significant association between expression of *GM-CSF* and number of mature DC in colorectal cancer.[32] A high level of TNF-*α* is known to induce migration of LHC to peripheral lymph nodes.[33] The association between high *TNF-α* expression with low numbers of intraepithelial LHC suggests that migration of LHC to peripheral lymph nodes occurred. This is in accordance with previous observations of Giannini *et al*,[27] who reported that increased *TNF-α* expression was associated with a reduced density of LHC in the transformation zone of the uterine cervix. Although high levels of *TNF-α* may lead to apoptosis, the presence of early HPV oncogene E6 presumably protects the cervical tumor cells from apoptosis by binding to the TNF-*α* receptor.[34]

IL-12p35 mRNA is produced ubiquitously by cervical cancer cells. It has been reported that, although there is a high expression of *IL-12p35* mRNA, due to preferential splicing, this does not result in protein expression unless *IL-12p40* is also present.[18] Production of IL-12p40 is much more restricted. It has been suggested that IL-12p40 plays a role in polarizing the immune response toward an antiviral response.[35]

Furthermore, low levels of IL-12 may support tumor growth. Our observations are consistent with a model whereby proinflammatory cytokines such as CCL2 and TNF-α attract monocytes to the tumor site. At the tumor site differentiation of TAM into mature DC occurs because of the presence of both GM-CSF and TNF-α. Adequate activation of TAM is prevented by high levels of active TGF-β.[36] DC seem to be pushed towards the T-regulatory state, thus preventing an adequate anti-tumor response. The high amounts of TNF-α presumably induce migration of resident LHC to peripheral lymph nodes.

Despite the migration of these APC to draining lymph nodes, this does not seem to result in an effective immune response, as shown by the lack of a significant correlation between the presence of TAM, mature DC, LHC, *TNF-α*, *GM-CSF*, and disease-free survival or cumulative overall survival. Although a previous study suggested that high TNF-α production was associated with an increased disease-free survival in non-small cell lung carcinoma, we did not find such an association.[37] A study by Ninck *et al* also found a relation between the expression of growth factors (such as GM-CSF) and disease-free survival, but not by GM-CSF alone.[38] Disease-free survival correlated with a poorer prognosis when head and neck squamous cell carcinoma simultaneously produced 3 or more growth factors. Paleri *et al* examined the production of IL-12 protein in head and neck squamous cancer.[39] Those authors did not find a significant correlation between IL-12 and disease-free survival. However, they did observe a trend that high production of the IL-12 protein was associated with an improved survival. Jones *et al* examined *IL-12p35* and *IL-12p40* expression in B-cell non-Hodgkin lymphoma.[40] In that study high *IL-12p40* levels were common in patients with a favorable prognosis. In our study no expression and high expression of *IL-12p40* correlated significantly with a favorable cumulative overall survival, whereas a low expression level of *IL-12p40* corresponded with a poorer cumulative overall survival. The similar results observed with a lack of *IL-12p40* expression and high *IL-12p40* expression may be related to the observation that IL-12p40 can form dimers. These IL-12p40 dimers have been reported to block the IL-12 receptor.[18] In both cases, lack of *IL-12p40* expression or high *IL-12p40* expression, a similar effect can be anticipated.

When the patients with and without post-operative radiotherapy were compared with respect to *IL-12p40* expression, it was observed that a higher amount of *IL-12p40* expression was significantly correlated with radiotherapy (*P*=0.030, data not shown). Therefore, the effect of IL-12p40 could be (partially) influenced by this clinicopathological parameter. Alternatively, a favorable cumulative overall survival of patients with a high expression level of *IL-12p40* can be explained by the capacity of this cytokine to increase the lytic activity and production of interferon-*γ* of natural killer cells and cytotoxic T-lymphocytes.[35] Elimination of target cells further requires migration of T-cells (and natural killer cells). Peritumoral stroma is essential for this migration.[41] Indeed, it was found that the percentage of stroma present in cervical carcinoma between no detectable *IL-12p40* and high *IL-12p40* versus low *IL-12p40* expression was decreased from 45% to 36%, respectively (*P*=0.020, data not shown), suggesting that an increased amount of stroma may facilitate T-cell migration.

Interestingly, an association between high expression of *IL-12p40* and high expression of TGF-*β* was observed (*P*=0.024). It has been shown that in cervical carcinoma there is a skewing in the direction of a T_H2 profile.[42] The presence of T_H2 cytokines (IL-4) is thought to enhance the development T_H3. T_H3 are known to produce substantial quantities of TGF-*β*. The association between high expression of *IL-12p40* and high expression of *TGF-β* may suggest that TGF-*β* either derived from T_H3 (tumor or stroma) may antagonize the effect of proinflammatory cytokines (e.g., IL-12p40) and subvert the cytokine profile in a direction that promotes tumor development.

The expression of different chemokines and cytokines such as CCL2, IL-12p40 TGF-*β*, and TGF-*β* target genes [e.g., plasminogen activator inhibitor (PAI)-1] are thought to reflect the condition of the tumor microenvironment. Although this knowledge will not have a direct effect on the present clinical management of cervical carcinoma, these parameters may be helpful to predict the further course of the disease. Furthermore, when HPV vaccines become available for the treatment of metastasized cervical carcinoma, the cytokine profile may be important to discriminate patients with a beneficial immune response from nonresponding patients.

In conclusion, the data presented in this study support the hypothesis that expression of cytokines by tumor cells is directed at attracting inflammatory cells to induce a smoldering inflammatory response that supports tumor growth and tumor

progression. However, a high expression level of *IL-12p40* accompanied by sufficient intratumoral stroma seems to tip the balance between tumor and inflammatory response and results in a more favorable cumulative overall survival.

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CHAPTER 4

Role of *IL-12p40* in cervical carcinoma

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ABSTRACT

Previously, we have shown that low *IL-12p40* mRNA expression by cervical cancer cells is associated with a poor survival of cervical cancer patients. Since IL-12p40 is both a subcomponent of IL-12 and IL-23, the aim of this study was to elucidate the role of IL-12p40 in cervical cancer. We have measured the expression of *IL-23p19* mRNA, *IL-12p35* mRNA and *IL-12p40* mRNA using mRNA *in situ* hybridization. IL-1 and IL-6 were measured by immunohistochemistry. Since IL-23 is a component of the IL-17/IL-23 pathway, a pathway induced by IL-1 and IL-6 in humans, we have studied IL-1 and IL-6 expression. Only a high number of stromal IL-6 positive cells was shown to associate with poor disease-specific survival. The worst disease-specific survival was associated with a subgroup of patients that displayed a high number of IL-6 positive cells and low *IL-12p40* expression ($P<0.001$). Both a high number of IL-6 positive cells and a high number of IL-6 positive cells plus low *IL-12p40* expression were shown to be clinicopathological parameters independent of lymph node metastasis, parametrial involvement and Sedlis score ($P=0.009$ and $P=0.007$, respectively). Our results with IL-6 and IL-12p40 are in accordance with the hypothesis that the IL-17/IL-23 pathway plays a suppressive role in cervical cancer.

INTRODUCTION

Cervical cancer is a leading cause of morbidity and mortality among women worldwide, especially in the developing countries.[1, 2] Infection with oncogenic types of human papillomavirus (HPV) is an important factor in the development of cervical cancer.[3, 4] The persistent HPV infection induces an inflammatory response. Inflammation is an important component in the majority of tumor types. The outcome of this inflammatory response surrounding the cancer cells is dependent on the composition of the inflammatory infiltrate and locally produced signaling molecules.[5] Although inflammatory cells within the neoplastic lesion are capable of generating an anti-tumor response this does not efficiently occur. [6, 7]

Inflammatory cells are attracted to the tumor site by locally produced cytokines and chemokines.[8, 9] Cervical cancer cells are known to produce an extensive range of cytokines and chemokines, such as CCL2, GM-CSF, TNF-α and IL-12.[10-12] In addition to attracting inflammatory cells, these cytokines and chemokines influence the activation status and function of infiltrating antigen presenting cells and stromal cells, thus influencing the course of the disease.[9, 13]

In a previous study we have shown that high expression levels or undetectable levels of *IL-12p40* mRNA in cervical carcinoma are associated with an improved overall survival compared to low amounts of *IL-12p40* that was associated with poor survival.[14] As IL-12 is known to stimulate effector cell populations such as cytotoxic T cells and natural killer cells,[15, 16] our results suggest a dual role for *IL-12p40.*

The IL-12 cytokine family includes IL-12, IL-23, IL-27 and IL-35.[17] From this family IL-12 and IL-23 share the IL-12p40 subchain. IL-12 is composed of IL-12p40 and IL-12p35 whereas IL-23 is composed of IL-12p40 and IL-23p19. IL-23 plays, amongst others, an important a role in the IL-17/IL-23 pathway resulting in the maintenance and expansion of T_H17 cells.[18] In addition to IL-23, IL-1 and IL-6 are thought to play an important role in the induction of T_H17 cells in humans.[19] The effect of IL-23 on cancer progression or cancer eradication is still not clear.[20, 21]

To further delineate the role of IL-12p40 in cervical carcinoma, we have quantified the mRNA expression levels of *IL-23p19* and compared its expression level with *IL-12p35 and IL-12p40* to investigate the relative importance of IL-12 and IL-23 in the

tumor microenvironment. In addition, we have investigated the role of IL-1 and IL-6 in the tumor microenvironment by determining both the number of IL-1 positive cells and the number of IL-6 positive cells. Finally, we have assessed the association between *IL-23p19, IL-12p35, IL-12p40*, high number of IL-6 positive cells and clinicopathological parameters.

MATERIAL AND METHODS

Patient material

Between 1985 and 1995, 254 untreated patients suffering from primary cervical carcinoma with stage IB and IIA underwent a radical hysterectomy type III with lymphadenectomy. From the tissue obtained, based on the availability of the material, 90 tissue samples were accessible for research. Tissues were routinely embedded in paraffin after 10% formalin fixation. The tissue samples of each patient were examined by a pathologist for the presence of tumor. Tumor percentage varied between 20% and 90%, median 60%. The characteristics of the patients are depicted in Table 1. Forty-seven patients received postoperative radiotherapy because of either tumor positive lymph nodes or the presence of positive risk factors described by the Sedlis criteria[22](a combination of 2 of the following unfavourable prognostic parameters: depth of infiltration ≥ 15 mm (deep stromal invasion; middle or deep third), tumor size ≥ 40 mm and presence of vasoinvasion). Human tissue samples were used according to the guidelines of the Ethical Committee of the Leiden University Medical Center.

Table 1. Summary of clinicopathological features of patients and tumors

Patient and tumor characteristics	Outcome	N*
Age	45 (mean)	90
	29 – 76 (range)	
FIGO	IB	68
	IIA	21
Lymph node metastasis	No	66
	Yes	22
Tumor size†	< 40 mm	37
	≥ 40 mm	26
Depth of infiltration†	< 15 mm	58
	≥ 15 mm	26
Vascular space involvement†	No	39
	Yes	47
Sedlis criteria•	Positive	28
	Negative	52
Parametrial invasion†	No	74
	Yes	14
HPV status‡	16, 18	66
	Other	15
Histology	Squamous	58
	Adenosquamous	18
	Adeno	7
	Other	6

* N=number of patients/cervical carcinomas.

† The number of reported cases is affected by incidental missing cases.

• Sedlis criteria{Sedlis, 1999 2345 /id}: a combination of 2 of the following unfavorable prognostic parameters: depth of infiltration ≥15 mm or >2/3 of the cervix, tumor size ≥ 40 mm and presence of vasoinvasion.

‡ Only data for cervical carcinoma samples with a determined HPV type were included. Other subtypes included HPV31 (n=2), HPV33 (n=6), HPV35 (n=1), HPV45 (n=3), HPV58 (n=1), HPV59 (n=2), HPV68 (n=1).

Preparation of *IL-12p35*, *IL-12p40* and *IL23p19*-probes

RNA was isolated from frozen human spleen using TRIzol® (Invitrogen™, Breda, The Netherlands) and first-strand cDNA was synthesized with oligoDT primers and Reverse Transcriptase AMV (both Roche Diagnostics, Mannheim, Germany), both according to manufacturer's instructions. Oligonucleotide primers for *IL23-p19*, *IL-12p35* and *IL-12p40* were chosen on the basis of known sequences (Table 2) and cDNA encoding for the different cytokines was amplified. A pGEM®-3Zf(+) Vector (Promega, Madison, WI) was linearized with SmaI and the PCR products were cloned

into the vector. After transferring the vector to *E. coli* strain Top 10 (Invitrogen Corp., San Diego, CA), the plasmids were isolated by using the QIAfilter Maxi KITS protocol (QIAGEN, Hilden, Germany). The sequence of the PCR product was confirmed by DNA sequencing. Plasmids were linearized with BamH1 and EcoR1 (both Boehringer, Mannheim, Germany) in case of *IL-12p40,* BamH1 and SacI in case of *IL-12p35* and SacII, SalI and SpeI (Boehringer) in case of *IL-23p19* using One-Phor-All Buffer Plus (Amersham Biosciences, Roosendaal, The Netherlands). Both strands were translated in a digoxigenin (DIG) labeled RNA probe according to manufacturer's instructions (Roche). The concentration of the DIG-labeled sense and antisense RNA probes were determined on a 1% agarose gel stained with ethidium bromide (Sigma, St. Louis, MO). Probes were stored at -20°C until further use.

RNA-*in situ* hybridization

The RNA-*in situ* hybridization (RISH) was performed as previously described.[23, 24] In short: 3 μm thick paraffin sections were pre-treated and hybridized with 100 ng/ml DIG labeled RNA probe diluted in hybridization mixture containing NaCl and saline-sodium citrate (SSC; Table 2). Hybridization was allowed for 16 hrs at either 55°C (*IL-23p19*) or 42°C (*IL-12p35* and *IL-12p40*), in a humidified chamber. Slides were washed 30 min in 2x SSC followed by 45 min in 0.1x SSC with 20 mM *β*-mercaptoethanol (Merck, Darmstadt, Germany), both at used hybridization temperature (Table 2). Subsequently the slides were incubated for 30 min with 2 U/ml ribonuclease (RNase) T1 (Roche) in 2x SSC, 1 mM EDTA at 37°C. RNA hybrids were detected using subsequently mouse anti-digoxigenin (1:2000, Sigma-Aldrich Chemie, Steinham, Germany), rabbit anti-mouse Ig (1:50, DAKO, Glostrup, Denmark) and mouse alkaline phosphatase anti-alkaline phosphatase(APAAP; DAKO).[10]

Table 2. RNA probes used and RNA-*in situ* hybridization conditions

Target	Primer	Sequence (5'-3')	Product (bp)	Accession number	Position	Hybridization Temperature ºC
IL-23p19	Forward Reverse	AGAGCCAGCCAGATTTGAGA GCAGATTCCAAGCCTCAGTC	487	NM_016584.2	134-620	55
IL-12p35	Forward Reverse	TGCTCCAGAAGGCCAGACAAAC CCCGAATTCTGAAAGCATGAAG	465	XM_003121	320-784	42
IL-12p40	Forward Reverse	GGACCAGAGCAGTGAGGTCTT CTCCTTGTTGTCCCCTCTGA	373	XM_004011	189-561	42

Immunohistochemistry

Serial sections, 3 μm thick, of formalin-fixed and paraffin-embedded tissue were mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinized, rehydrated and treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity.

Antigen retrieval was performed (0.01 M citrate, pH 6.0) and sections were rinsed in phosphate-buffered saline (PBS). Subsequently, sections were stained overnight using either a 1:100 dilution of an affinity purified polyclonal goat anti-human IL-1β antibody (AF-201-NA; R&D Systems, Minneapolis, MN) or a 1:300 dilution of anti-human polyclonal rabbit anti-IL-6 antibody (Abcam, Cambridge, UK). For anti-IL-1 the slides were incubated with a goat HRP-polymer kit (Biocare Medical, Concord, CA) according to the manufacturer's instructions. For anti-IL-6 the slides were incubated with respectively a biotinylated swine anti-rabbit antibody (1:200; DAKO) and subsequently incubated with a biotinylated horseradish peroxidase–streptavidin complex (1:100; DAKO). Immune complexes were visualized with diaminobenzidine as previously described.[24]

(Semi-)quantitative evaluation of RISH and immunohistochemistry

RISH was scored as previously described.[25] Intensity was scored as none (0), mild (1), moderate (2) or intense (3) at low magnification (100x). Furthermore, the percentage of positive tumor cells was determined and divided in six groups: 0% (0, absent), 1-5% (1, sporadic), 6-25% (2, local), 26-50% (3, occasional), 51-75% (4, majority) and 76-100% (5, large majority). The sum of both the percentage and the staining intensity of the positive cells resulted in an overall score (0 or 2 to 8). The scores were combined into three groups: category 0 (score 0, no expression), category 1 (scores 2, 3, 4 and 5, low expression) and category 2 (scores 6, 7 and 8, high expression). mRNA expression was scored by two independent researchers without knowing the identity and clinical outcome of patients. IL-1 positive cells and IL-6 positive cells were quantified in the tumor by counting the number of stained cells per 6, randomly selected, high-power field of view (HPF, 400x).

Statistical analysis

Data from immunohistochemistry as well as RISH are given as the mean ± the SD. Statistical analysis was done using SPSS 17.0 (SPSS Inc., Chicago, IL). Data were processed by using the chi-square test. Kaplan-Meier survival curves were generated to assess differences in disease-free period (defined as the observation time in months from surgery to relapse of the disease (disease-free survival)) or cumulative disease-specific survival (defined as time in months from surgery to death due to cervical cancer). A Cox regression was used for multivariate survival analysis. $P<0.05$ was considered statistically significant.

RESULTS

Patients

Of the group of 90 patients, 68 patients were diagnosed as FIGO stage IB and 21 as FIGO stage IIA and all underwent radical hysterectomy combined with pelvic lymph adenectomy (Table 1). Forty-seven patients received postoperative radiotherapy because of either tumor positive lymph nodes or meeting the terms of the Sedlis criteria[22] (a combination of 2 of the following unfavorable prognostic parameters: depth of infiltration ≥ 15 mm, tumor size ≥ 40 mm and presence of vasoinvasion). Twenty five patients suffered from recurrent disease. At the end of the study seventy patients were alive, 7 suffered from a recurrence and 18 patients had died of disease.

Expression of *IL-23p19*, *IL-12p35* and *IL-12p40* in cervical cancer

Since IL-12p40 is both a subunit of IL-12 as well as IL-23, we have determined the expression of *IL-23p19.* Both of *IL-23p19* and *IL-12p40* were expressed by cervical tumor cells (Figure 1A and Figure 1C). The expression of *IL-12p40* was stronger than the expression of *IL-23p19. IL-23p19* was expressed in 63% of the samples (n=54), *IL-12p40* was expressed in 54% of the samples (n=90) and *IL-12p35* was expressed in 84% of the samples (n=90; Table 3). All samples that expressed either *IL-23p19* or *IL-12p40* also expressed *IL-12p35* mRNA. In contrast, 13 out of 44 samples that expressed *IL-12p40* did not express *IL-23p19.*

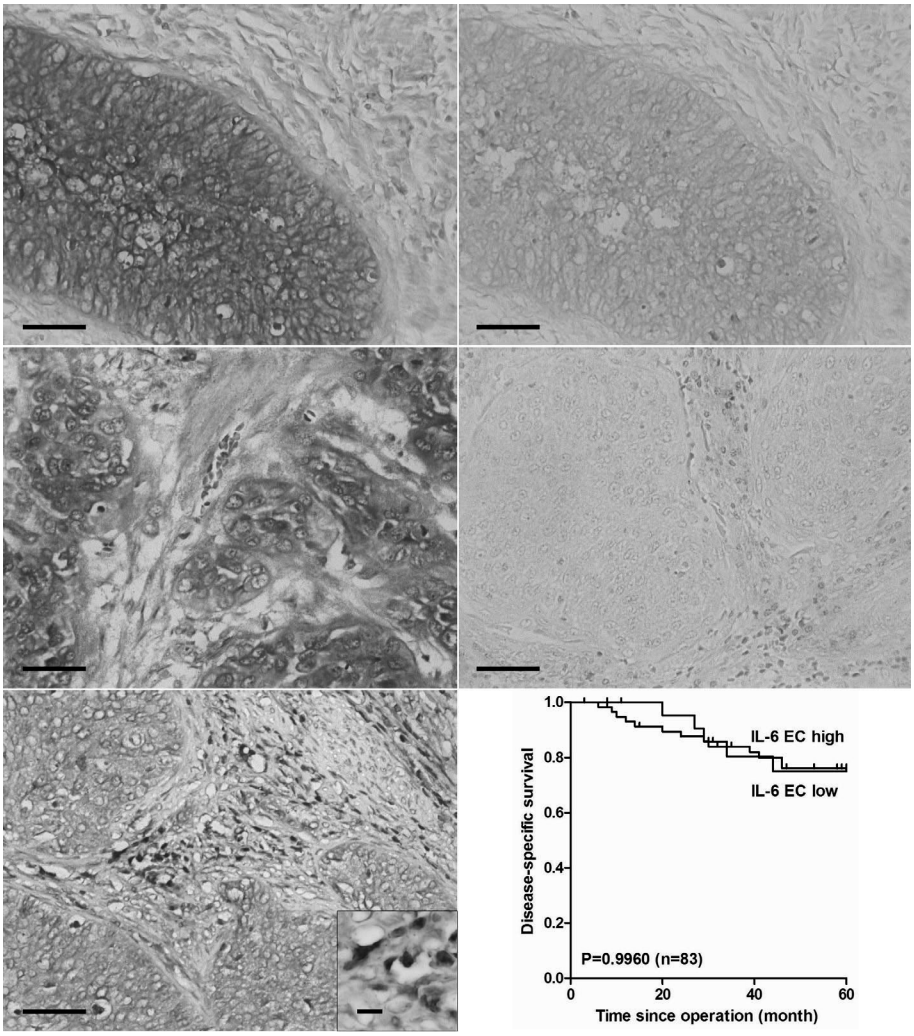

Figure 1 The expression of *IL-23p19*, *IL-12p40* and IL-6. The expression of *IL-23p19* and *IL-12p40* were determined using RNA-*in situ* hybridization (RISH) and the expression of IL-6 was determined using immunohistochemistry as described in the Materials and methods section (magnification, x250). (**A**) Cervical tumor, *IL-23p19* RISH. Tumor cells stain positive (moderate) for *IL-23p19*; (**B**) Negative (sense) control of *IL-23p19* RISH; (**C**) Cervical tumor, *IL-12p40* RISH. Tumor cells stain positive (strong) for *IL-12p40;* and (**D**) Negative (sense) control of *IL-12p40* RISH. (**E**) IL-6 staining of cervical cancer tissue. Both cells in the epithelial compartment (EC) as well as cells in the stroma express IL-6. Arrows indicate positive stromal cells. Detail (x400, magnification) of IL-6 positive cells in the stroma; and (**F**) Association between cells in the epithelial compartment with low (IL-6 EC low) and high IL-6 (IL-6 EC high) expression and disease-specific survival. No significant association between low or high IL-6 expression of the epithelial cells with disease-specific survival was observed. *See page 193 for color figure.*

A positive correlation between *IL-23p19* and *IL-12p40* was found (n=54, r^2=0.117, *P*=0.011; data not shown). No statistically significant correlation between *IL-23p19* and *IL-12p35* was found (n=54, r^2=0.061, *P*=0.072; data not shown).

Table 3. Correlation between *IL-23p19, IL-12p35* and *IL-12p40* expression in cervical carcinoma

		IL-12p35			
Cytokine	Expression level	Absent	Low	High	Total N* (%)
IL-12p40	Absent	14	21	6	41 (46)
	Low	0	14	14	28 (31)
	High	0	3	18	21 (23)
Total	N* (%)	14 (16)	38 (42)	38 (42)	90 (100)
P-value					**<0.001**
		IL-23p19			
		Absent	Low	High	Total N* (%)
IL-12p40	Absent	7	1	2	10 (19)
	Low	8	5	11	24 (44)
	High	5	4	11	20 (37)
Total	N* (%)	20 (37)	10 (19)	24 (44)	54 (100)
P-value					0.188
		IL-23p19			
		Absent	Low	High	Total N* (%)
IL-12p35	Absent	1	0	0	1 (2)
	Low	8	5	8	21 (39)
	High	11	5	16	32 (59)
Total	N* (%)	20 (37)	10 (19)	24 (44)	54 (100)
P-value					0.619

*N=number of tumors. The scores were combined into three groups: absent expression, low expression and high expression as described in the Materials and methods section. Statistical significant *P*-values are bold.

Association between IL-12 and IL-23 and disease-specific survival in cervical cancer

To investigate the relationship between the expression of *IL-23p19, IL-12p35* and *IL-12p40*, and disease-specific survival, Kaplan Meier plots were created. A log-rank test was used to determine statistical differences in disease-specific survival. Since absence of *IL-12p40* will result in neither IL-12 nor IL-23, we first confirmed that expression of *IL-12p40* was associated with poor disease-specific survival (Figure 2A; n=48, log-rank test 5.753, *P*=0.017) in this cohort. The expression of *IL-12p35* (Figure 2B; n=74, log-rank test 0.2019, *P*=0.653) and the expression of *IL-23p19* (Figure

2C; n=33, log-rank test 1.930, *P*=0.165) were both not significantly associated with disease-specific survival. The expression of *IL-23p19, IL-12p35* or *IL-12p40* showed no significant difference in disease-free survival (data not shown).

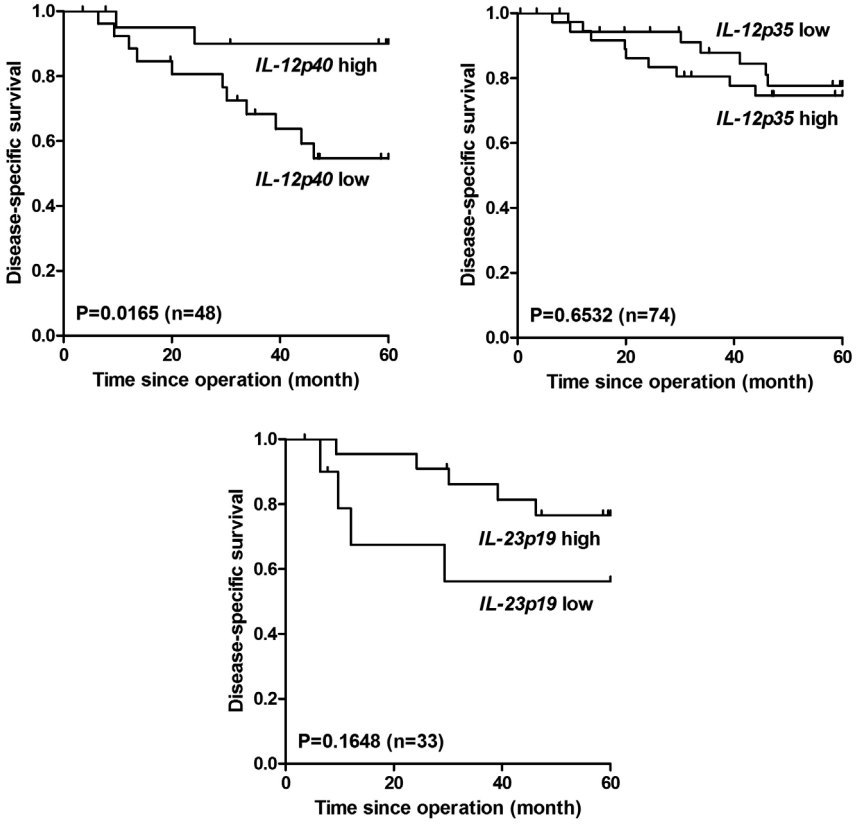

Figure 2 Association between (**A**) *IL-12p40,* (**B**) *IL-12p35* and (**C**) *IL-23p19* expression and disease-specific survival in cervical carcinoma. *IL-12p40, IL-12p35* and *IL-23p19* were determined by RISH as described in the Materials and methods section.

Presence of IL-6 positive cells and association with disease-specific survival in cervical cancers

The presence of *IL-23p19* suggests that IL-23 may sustain a T_H17 positive cell population in cervical cancer. Since differentiation towards the IL-17/IL-23 pathway is thought to occur in the presence of IL-1 and IL-6 in humans,[19] we have determined

the presence of IL-1 positive cells and IL-6 positive cells, using immunohistochemistry. IL-1 was predominantly expressed by cells in the stromal compartment. Occasionally tumor cells also showed weak IL-1 expression. No statistical significant association between low or high number of IL-1 expressing cells and disease-specific survival was observed (Figure 3A). IL-6 was expressed by both cells in the epithelial (tumor cell) compartment as well as cells in the stromal compartment (Figure 1E). No significant association was observed between low or high IL-6 expression of cells in the epithelial compartment and disease-specific survival (Figure 1F). Subsequently, we quantified the number of IL-6 positive cells in the stroma. The presence of a high number of IL-6 stromal positive cells (median 17 IL-6 positive cells/high powered field) significantly associated with disease-specific survival (Figure 3B; n=83, log-rank test 12.57, $P<0.001$). No statistical significant difference was observed for disease-free survival (data not shown). We also determined whether disease-specific survival was associated with the presence of both a high number of IL-6 positive stromal cells and low *IL-12p40*. In this latter case an even stronger decrease in disease-specific survival was observed (Figure 3C; n=47, log-rank test 20.38, $P<0.001$).

Association between low IL-12p40 expression, high number of IL-6 positive stromal cells and clinicopathological parameters

To determine the relevance of our findings we associated our immunological findings with clinicopathological parameters. First a univariate Cox analysis was performed, using the clinical parameters, Sedlis criteria (two out of three of the following criteria positive: tumor size ≥ 40 mm, vasoinvasion and deep stromal invasion), lymph node metastasis and parametrial involvement and the immunological parameters, low *IL-12p40* expression, high number of IL-6 positive cells and high number of IL-6 positive cells plus low *IL-12p40* expression. In the univariate Cox analyses all the included parameters showed a significantly increased hazard ratio (Table 4). Subsequently, a multivariate Cox analysis with the three clinicopathological parameters and each of the significantly immunological parameters was performed. In this case two of the three immunological parameters, high number of IL-6 positive stromal cells ($P=0.009$; HR 7.447) and high number of IL-6 positive stromal cells and low *IL-12p40* expression ($P=0.007$; HR 20.123) were shown to be independent predictors of poor disease-specific survival.

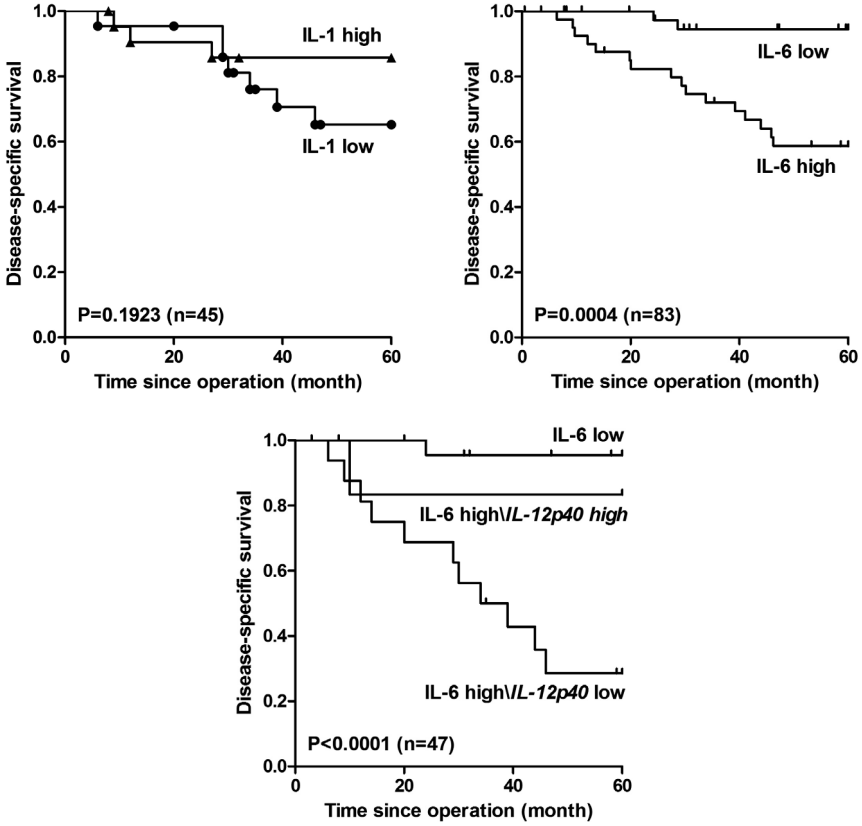

Figure 3 Association between high number of IL-1 positive cells, high number of IL-6 positive stromal cells, low *IL-12p40* expression and disease-specific survival. The IL-1 expression and IL-6 expression were determined by immunohistochemistry as described in Materials and methods. *IL-12p40* was determined by RISH as described in the Materials and methods section.

Table 4. Cox regression of clinicopathological variables and *IL-12p40*, *IL-23* and the number of IL-6-positive cells in cervical carcinoma

Univariate	**Hazard ratio**	**95% CI**	***P*-value**
Sedlis positive	4.354	1.608-11.789	**0.004**
Lymph node metastasis	3.266	1.286-8.296	**0.013**
Parametrial involvement	3.645	1.411-9.411	**0.008**
Low *IL-12p40*	5.231	1.156-23.661	**0.032**
High number of IL-6 cells	8.975	2.063-39.051	**0.003**
Low number of IL-6 cells	Reference		
High number of IL-6 cells/low *IL-12p40*	21.832	2.805-169.915	**0.003**
Multivariate	**Hazard ratio**	**95% CI**	***P*-value**
Sedlis positive	3.848	1.270-11.663	**0.017**
Lymph node metastasis	1.333	0.439-4.052	0.612
Parametrial involvement	1.526	0.542-4.444	0.438
High number of IL-6 cells	7.447	1.659-33.432	**0.009**
Multivariate	**Hazard ratio**	**95% CI**	***P*-value**
Sedlis positive	0.706	0.150-3.310	0.659
Lymph node metastasis	2.887	0.836-9.965	0.094
Parametrial involvement	1.168	0.283-4.822	0.830
Low number of IL-6 cells	Reference		
High number of IL-6 cells/low *IL-12p40*	20.123	2.248-180.147	**0.007**

Shown are the log-rank test and *P*-value of compared expression levels.
Statistical significant *P*-values are bold.

DISCUSSION

In a previous study we found an association between low expression of *IL-12p40* and poor disease-specific survival, whereas high expression of *IL-12p40* or lack of expression of *IL-12p40* were associated with a favorable disease-specific survival.[14] Since IL-12p40 combines with both IL-12p35 and IL-23p19, to form IL-12 and IL-23, respectively, in the present study, we have further investigated the role of *IL-12p40* in cervical cancer.

Both *IL-23p19* and *IL-12p35* were expressed in the majority of the samples. Out of the 44 samples that expressed *IL-12p40, 13* samples did not express *IL-23p19.* Since *IL-12p35* expression seems to be ubiquitous in cervical cancer,[14] the level of *IL-12p40* or *IL-23p19* expression most probably determines whether IL-12, IL-23 or

both are expressed. In our study, we observed a trend (*P*=0.061; n=54) between *IL-23p19* or *IL-12p35* expression, whereas in the study of Wolf *et al* in ovarian cancer (n=112) a significant correlation between the expression of *IL-23p19* and *IL-12p35* was found.[26] The discrepancy between our results and the results of Wolf *et al* may be due to the smaller size of our study group. Very few studies have investigated the association between local expression of IL-12 or IL-23 and prognosis. Using immunohistochemistry, IL-12 has been associated with improved survival in patients with (advanced) gastric carcinoma.[27, 28] In the study of Wolf *et al*, using RT-PCR, both *IL-12p35* and *IL-23p19* were associated with a superior outcome.[26] In a multivariate analysis *IL-12p35* was found to be an independent factor for overall survival of ovarian carcinoma. As stated previously, we have observed a statistically significant association between low expression of *IL-12p40* and poor disease-specific survival in cervical carcinoma.[14] In the present study and our previous study,[14] we did not find a significant association between either *IL-23p19* or *IL-12p35* expression and disease-specific survival. Since *IL-23p19* and *IL-12p35* are both expressed, it is important to determine which cytokine, IL-23 or IL-12, has a dominant effect on the tumor microenvironment.

The molecular interaction between IL-23p19 and IL-12p40 has been studied by Beyer *et al*.[29] These authors reported that the interface region of IL-23p19 and IL-12p35 on IL-12p40 overlap. Due to different interresidue interactions of IL-12p35 and IL-23p19 with IL-12p40, these molecules interact with a different affinity with IL-12p40. Therefore the availability of IL-12p40 in combination with the affinity for IL-12p35 and IL-23-p19 may result in skewing of the IL-12/IL-23 response. This is supported by experiments performed by Zwiers *et al*. These authors showed that in an experimental animal model polymorphic variants of IL-12p40 can skew IL-12/IL-23 synthesis.[30] Thus both differences in protein interactions between IL-23p19 and IL-12p35 on the one hand and IL-12p40 on the other hand and genetic polymorphisms in the protein chains, such as IL-12p40, contribute to the amounts of IL-12 and IL-23 formed. Our results support a previously suggested immunosuppressive role for IL-23.[20] This is further supported by a study in ovarian carcinoma were genetic differences in the IL-23 receptor have been reported to influence prognosis.[31]

In contrast, it has also been shown that overexpression of IL-23 reduces tumor growth and metastasis formation and that IL-23 is able tot elicit a strong cytotoxic T-cell memory response,[21, 32] underscoring our view that the level of expression of the different cytokines and chemokines plays an import role in the final outcome.

Our data suggest that in the presence of a limited amount of *IL-12p40* the biological effect of IL-23 dominates, whereas in the presence of a high amount of *IL-12p40* the biological effect of IL-12 prevails. Since IL-12 polarizes the immune response towards an anti-viral response,[33] the favorable cumulative overall survival of patients with a high IL-12 positive cell density,[27, 28] high expression level of *IL-12p35*[26] and *IL-12p40*[14] can be explained by the capacity of this cytokine to increase the lytic activity and the production of interferon-*γ* of natural killer cells and cytotoxic T-lymphocytes.[33] Interestingly, we previously observed an association between high expression of *IL-12p40* and high expression of *TGF-β* (P=0.024),[14] suggesting that the tumor cells are selected to counter act the effect of IL-12 or skew the response to the IL-17/IL-23 pathway.

In humans, in addition to IL-1, IL-6 and TGF-*β* have been implicated to play a role in the IL-17/IL-23 pathway.[19, 34] Previously, we have shown that it is likely that activated TGF-*β* is present in the tumor microenvironment, since PAI-1, a target gene of TGF-*β*, is expressed and expression of PAI-1 is associated with survival.[35] Furthermore, both the integrin *αvβ*6 and active matrix metalloproteinase-2, known to activate TGF-*β*, are associated with poor disease-specific survival.[36, 37] Even though the role of TGF-*β* in inducing T_H17 in humans has been questioned, TGF-*β* may suppress T_H1 and T_H2 development thus favoring T_H17 development.[38, 39]

In our study, low or high numbers of IL-1 positive cells were not associated with disease-specific survival. The presence of a high number of IL-6 expressing stromal cells was significantly associated with poor disease-specific survival (P<0.001). Previously, IL-6 has been implicated as an autocrine or paracrine growth factor for cervical cancer.[40, 41] IL-6 has been shown to induce VEGF transcription via the STAT3 signaling pathway, thus promoting an angiogenic switch.[42] Indeed, blockade of the IL-6 receptor on cervical cancer cell lines was shown to interfere with cell survival signals and blocked expression of VEGF.[43]

As HPV vaccines will become available for the treatment of metastasized cervical carcinoma, the local cytokine/chemokine profile may be important to discriminate patients with a beneficial immune response from non-responding patients.
In conclusion, *IL-12p40* plays at least a dual role in cervical carcinoma by associating with both *IL-23p19* and *IL-12p35*. We have shown that low *IL-12p40* expression was significantly associated with poor disease-specific survival (P=0.017). Also a high number of stromal IL-6 producing cells was shown to associate with poor disease-specific survival (P<0.001). The worst disease-specific survival was observed in a subgroup of patients that displayed a high number of stromal IL-6 expressing cells and low *IL-12p40* expression (P<0.001). Furthermore, both a high number of stromal IL-6 expressing cells and a high number of stromal IL-6 plus *IL-12p40* expression were shown to be independent clinicopathological parameters compared to lymph node metastasis, parametrial involvement and Sedlis score (P=0.009 and P=0.022 respectively). Our results with IL-6 and IL-12p40 are in accordance with the hypothesis that the IL-17/IL-23 pathway plays a suppressive role in cervical cancer.

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CHAPTER 5

Expression of endoglin (CD105) in cervical cancer

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ABSTRACT

In this study, we have investigated the role of endoglin (CD105), a regulator of transforming growth factor (TGF)-β_1 signaling on endothelial cells, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)-A in cervical cancer. We have measured the number and determined the location of both newly formed (CD105-positive) and the overall number of (CD31-positive) blood vessels, and bFGF and VEGF-A expression using immunohistochemistry in 30 cervical carcinoma specimens. Vascular endothelial growth factor-A mRNA expression was determined using RNA-*in situ* hybridization. CD105- and CD31-positive vessels and bFGF- and VEGF-A-positive cells were predominantly present in the stroma. The presence of CD105- and CD31-positive vessels in the stroma did neither correlate with the number of VEGF-A-positive cells nor the number of bFGF-positive cells. However, the number of CD105- and CD31-positive vessels was associated with the expression of *VEGF-A* mRNA in the epithelial cell clusters (P=0.013 and P=0.005, respectively). The presence of CD105-positive and CD31-positive vessels was associated with the expression of $\alpha v\beta 6$ (a TGF-β_1 activator; P=0.013 and P=0.006, respectively). Clinically, the number of CD105-positive vessels associated with the number of lymph node metastasis (P<0.001). Furthermore, the presence of CD105-positive vessels within the epithelial cell clusters associated with poor disease-free survival (P=0.007).

INTRODUCTION

Cervical cancer is the second most common cancer among women worldwide and one of the main causes of cancer-related death in the developing countries.[1] The prognosis of patients is, amongst others, dependent on tumor size, lymph node metastasis, capillary lymphatic space involvement, infiltration of the tumor in the cervix, infiltration of the tumor in the parametria and absence of tumor at the surgical margins.[2] Cervical tumors are composed of malignant epithelial cells, tumor stroma, comprising the tumor vasculature, and an inflammatory infiltrate. A significant association between tumor vascular density and poor clinical outcome has been reported.[3-5] Growth and metastatic capacity of solid tumors depend on tumor vascular density and angiogenesis.[6, 7] Hypoxia in the centre of the epithelial cancer cell nests is thought to be a driving force behind angiogenesis.[8]

Tumors promote angiogenesis by secreting factors, like vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (bFGF)[7, 9] and transforming growth factor (TGF)-β_1. In addition, the presence of an inflammatory infiltrate is thought to be of great importance in neovascularisation.[10]

A marker for angiogenesis is endoglin.[11] Endoglin, designated CD105, is a 90 kDa (under reducing conditions) TGF-β type III auxiliary receptor involved in the regulation of TGF-β_1 signaling in endothelial cells. Overexpression of CD105 inhibits TGF-β/ALK5 signaling and TGF-β-induced growth inhibition, whereas knockdown of CD105 inhibits TGF-β/ALK1 signaling and endothelial cell proliferation.[12] CD105 is required for efficient TGF-β/ALK1 signaling on proliferating endothelial cells.[13] In contrast to CD31, which is expressed on blood vessels both in normal tissue and in malignant lesions, endoglin is found predominantly on peritumoral and intratumoral blood vessels. The expression of CD105 on tumor-associated blood vessels makes CD105 a potential molecular target for therapy.[11]

TGF-β_1 plays a dual role in cancer: early in tumor development it acts as a tumor suppressor, inhibiting epithelial cell proliferation, whereas late in cancer development it suppresses the activity of the immune system and induces regulatory T cells.[14] TGF-β_1 also plays an important role in angiogenesis by promoting proliferation and migration of endothelial cells at low TGF-β_1 concentrations, whereas high concentrations lead

to cytostasis and promote vessel maturation.[13] Furthermore, TGF-β_1 is known to induce VEGF expression.[15] Earlier, we have reported on the expression of TGF-β_1, plasminogen activator inhibitor (PAI)-1 and fibronectin (both target gene products of TGF-β_1) and $\alpha v\beta 6$ (activator of TGF-β_1) in cervical carcinoma. [16-18]

In this study, we have expanded our observations on the role of TGF-β_1 in cervical cancer by measuring the number and determining the location of CD105-positive blood vessels. To further analyze the role of endoglin as an TGF-β_1 associated regulatory molecule in the angiogenic process, we have compared the number and location of the newly formed CD105-positive vessels with the number and location of CD31-positive blood vessels (total number of blood vessels) and we have measured the expression of the pro-angiogenic factors, VEGF-A and bFGF. Finally, the clinicopathological relevance of CD105 in cervical carcinoma was assessed.

MATERIALS AND METHODS

Patient material

A total of 30 patients treated with radical abdominal hysterectomy and bilateral pelvic lymph node dissection for uterine cervical cancer (Wertheim-Meigs procedure) was included in this study. According to the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) staging system for cervical carcinoma[19] 10 patients were selected for FIGO stage IIA or more, whereas the other 20 patients were randomly selected patients with a FIGO stage IB. Patients had received no therapy before surgery. Treatment occurred between 1985 and 1994. Tissues had been fixed routinely in 10% v/v formalin and embedded in paraffin. Samples were used according to the guidelines of the Ethical Committee of the Leiden University Medical Center. The patient characteristics are shown in Table 1.

Table 1. Summary of clinicopathological features of patients and tumors

Characteristics of patients and tumors	Outcome	N*
Age	45, mean	30
	29 – 72, range	
FIGO stage	≤IB	20
	≥IIA	10
Lymph node metastasis	No	16
	Yes	14
Tumor size†	<40 mm	16
	≥40 mm	12
Infiltration depth†	<15 mm	15
	≥15 mm	9
Vascular space involvement	No	10
	Yes	20
Parametrial invasion	No	20
	Yes	10
HPV status†	16, 18	19
	Other	6
Histology	Squamous	25
	Adenosquamous	4
	Adeno	1

* N=number of patients/cervical carcinomas.
† Cases missing.

Immunohistochemistry

Immunohistochemical analysis was performed on 3 μm paraffin sections, mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinized, rehydrated and treated with 0.3% v/v H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. Antigen retrieval was performed, if necessary, and sections were rinsed in phosphate-buffered saline (PBS) followed by an incubation with 1% w/v bovine serum albumin (BSA) in PBS. Subsequently, sections were stained for CD68,[20] *αvβ6*,[16] fibronectin,[18] VEGF-A, bFGF, matrix metalloproteinase (MMP)-2,[21] CD31 and CD105 (see Table 2 for characteristics of the primary antibodies). All antibodies were diluted in 1% BSA in PBS. In case of CD68, *αvβ6*, bFGF, MMP-2 and CD31 a biotinylated secondary rabbit anti-mouse antibody was used (1:200, DAKO, Glostrup, Denmark). For VEGF-A, a biotinylated swine anti-rabbit antibody, and for fibronectin, biotinylated rabbit anti-goat antibody was used (both 1:400, DAKO). All slides

were subsequently incubated with a biotinylated horseradish peroxidase (HRP)-streptavidin complex (1:100, DAKO) and immune complexes were visualized with diaminobenzidine. Staining for CD105 was performed with a CSA Detection System (DAKO), according to the manufacturer's protocol. Negative controls consisted of tissue sections where the primary antibody was replaced by an (irrelevant) antibody (DAKO) directed against the same isotype as the primary antibody.

Table 2. Characteristics of used primary antibodies

Antigen	Clone	Source	Directed against	Antigen retrieval	Dilution	Incubation conditions	Manufacturer
αvβ6	2G2	Mouse	*β6*	Citrate 0.01 M	1:2000	on* 4°C	Biogen Idec, Cambridge, MA
bFGF	6	Mouse	bFGF	Citrate 0.01 M	1:600	on 4°C	BD Biosciences, Franklin Lakes, NJ
CD31	JC/70A	Mouse	Endothelial cells	Citrate 0.01 M	1:400	on RT	Neomarkers, Fremont, CA
CD68	KP-1	Mouse	Macrophages	Trypsin 0.1% w/v	1:1600	on RT†	DAKO, Glostrup, Denmark
CD105	SN6H	Mouse	Endoglin	None	1:2000	1 h RT	DAKO, Glostrup, Denmark
Fibronectin		Goat	Fibronectin	Pepsin 0.4% w/v	1:1000	on RT	Sigma, St. Louis, MO
MMP-2	CA-4001	Mouse	Proform of MMP-2	None	1:200	on RT	Neomarkers, Fremont, CA
VEGF-A		Rabbit	VEGF-A	Pepsin 0.4% w/v	1:100	2 h RT	Santa Cruz Biotechnology Inc., Santa Cruz, CA

bFGF = basic fibroblast growth factor; MMP-2 = matrix metalloproteinase-2;VEGF-A = vascular endothelial growth factor-A.
*on = overnight
†RT = room temperature

Probe preparation and RNA-*in situ* hybridization

Oligonucleotide primers were chosen on the basis of known sequences [5' GCCTCCGAAACCATGAACTTT 3' (sense) and 5' CCGCATAATCTGCATGGTGAT 3' (antisense)] (Gen Bank accession number GI065522.1).

Cervical carcinoma sections were stained for V*EGF-A* mRNA as previously described.[20] In short: 3 μm paraffin sections were pre-treated and hybridized with 100 ng/ml digoxigenin (DIG)- labeled RNA probe diluted in hybridization mixture

containing 0.3 M NaCl and 0.03 M saline-sodium citrate (SSC). Hybridization was allowed for 16 h at 50°C in a humidified chamber. Slides were washed for 30 min in 50% v/v formamide/2x SSC at 42°C, followed by 45 min in 0.1x SSC with 20 mM *β*-mercaptoethanol at 50°C and 30 min with 2 U/ml ribonuclease (RNase) T1 (Roche Diagnostics, Mannheim, Germany) in 2x SSC, 1 mM EDTA at 37°C. RNA hybrids were detected using subsequently mouse anti-digoxigenin (1:2000, Sigma-Aldrich Chemie, Steinham, Germany), rabbit anti-mouse Ig (1:50, DAKO) and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP, DAKO). The sense probe of each antisense V*EGF-A* mRNA probe served as a negative control. A cervical cancer sample stained for *TGF-β1* mRNA served as a positive control.

Evaluation of immunohistochemical staining and RNA-*in situ* hybridization

CD68-, VEGF-A- and bFGF- positive cells were quantitated by counting the number of stained cells per five, randomly selected, high-power fields of view (HPF, x400). CD68-positive cells at the border of the epithelial-stromal interface were counted as present or absent. CD31- and CD105-positive vessels were quantitated by first searching for a high density of vessels at low magnification (x200, the so called "hot-spots") in the tumor stroma, followed by counting the number of positive vessels in five of these hot-spots at high magnification (HPF, x400).[22] The presence of CD105-positive vessels within the epithelial cell clusters or at the epithelial-stroma interface, were counted as present or absent. Fibronectin was scored at the epithelial-stroma interface as described by Havenith *et al*,[23] dividing the immunoreactivity in either <75% immunoreactivity or >75% immunoreactivity.

The staining of *VEGF-A* mRNA as well as VEGF-A protein, PAI-1, MMP-2 and *αvβ*6 in the tumor cells was scored as described earlier.[24] Intensity was scored as none (0), weak (1), moderate (2) or strong (3) at low magnification (x100). Furthermore, the percentage of positive tumor cells was determined and divided into groups, numbered from 0 to 5: 0% (0, absent), 1% to 5% (1, sporadic), 6% to 25% (2, local), 26% to 50% (3, occasional), 51% to 75% (4, majority) and 76% to 100% (5, large majority). The two parameters were combined, representing the sum of both the percentage and the staining intensity of the positive cells, resulting in an overall score (0 or 2-8). Owing to low expression of *VEGF-A*, the scores were combined into

two groups: category 0 (score 0, no expression) and category 1 (score 2-8, expression present). Expression was scored by two independent researchers without knowing the identity and clinical outcome of patients.

Statistical analysis

Data from immunohistochemistry as well as RNA-*in situ* hybridization are given as the mean ± the standard deviation (SD). Statistical analysis was performed using SPSS 14.0 (SPSS Inc., Chicago, IL). Data were processed by using a chi-square test, the Mann-Whitney *U*-test or the Fisher's exact test, depending on number and distribution of the compared groups. Kaplan-Meier survival curves were generated to assess differences in disease-free survival (defined as the observation time in months from surgery to relapse of the disease) or cumulative overall survival (defined as time in months from surgery to death owing to cervical cancer). $P<0.05$ was considered statistically significant.

RESULTS

Number and location of CD105- and CD31-positive blood vessels

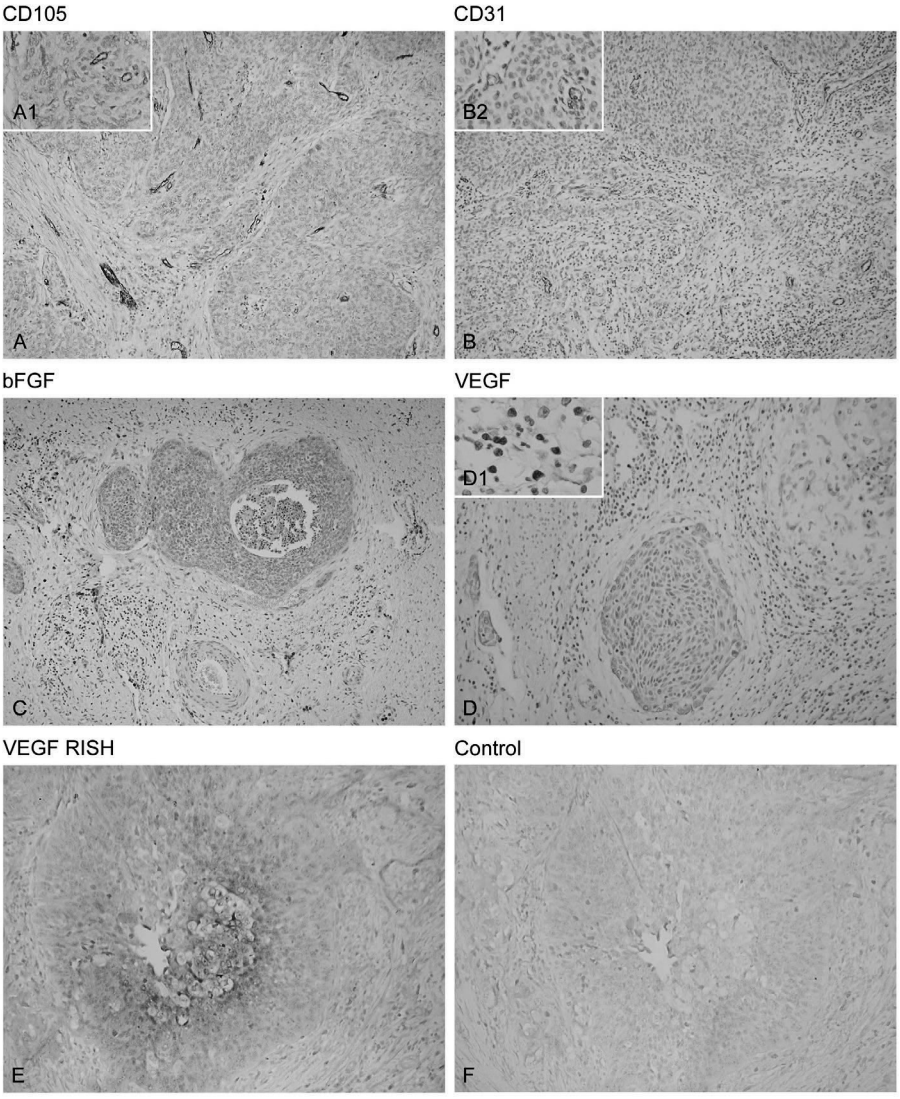
First, we have investigated the relationship between the number and location of (newly formed) blood vessels, using anti-CD105 and anti-CD31 (providing an estimate of the total number of blood vessels) monoclonal antibodies (Figure 1A and 1B, respectively). The number of newly formed stromal CD105-positive vessels (mean 5 ± 1) was lower than the number of stromal CD31-positive vessels (mean 9 ± 1). The number of CD105-positive vessels ranged from 0 to 20, whereas the number of CD31-positive vessels ranged from 2 to 23. In 8 out of 30 cases CD105-positive vessels were located within the epithelial cell clusters, whereas in 18 out 30 cases CD31-positive vessels were observed within these clusters. There was a significant correlation between the expression of CD105- and CD31-positive vessels in the stroma ($r^2=0.747$, $P<0.001$; Figure 2).

Number and location of VEGF-A and bFGF-positive cells

Subsequently, we have investigated the number and location of bFGF- and VEGF-A-positive cells (Figure 1C and 1D, respectively). Basic fibroblast growth factor-positive cells were predominantly observed in the stroma. The number of bFGF-positive cells ranged from 0 to 71 (mean 24 ± 5). Only 3 out of 30 cases showed bFGF expression within the epithelial cell nests. These cells were mainly located at the epithelial-stromal interface. Another 6 tumors showed bFGF positive cells within the necrotic centers of epithelial nests, whereas tumor cells showed a negative staining. VEGF-A was both measured at the protein level using immunohistochemistry and at the mRNA level using RNA-*in situ* hybridization (Figure 1E). VEGF-A positive cells were mainly observed in the stroma. The number of VEGF-A positive cells ranged from 0 to 77 (mean 11 ± 3). Weak VEGF-A protein expression was observed throughout the epithelial cell clusters with an increased intensity at the epithelial-stromal interface (16 out of 30 cases; Figure 1D). In addition, *VEGF-A* (mRNA) expression was observed in the epithelial cell clusters (14 out of 30 cases). Expression of *VEGF-A* (mRNA) within the epithelial cell clusters did not correlate with VEGF-A protein expression within the epithelial cell clusters, but did associate with the number of VEGF-A positive cells in the stroma (*P*=0.048, Figure 3A).

Association between blood vessels, bFGF and VEGF-A

No significant association between the presence of CD105-positive vessels in the tumor or the total number of CD105-positive and CD31-positive vessels in the stroma, and the number of bFGF- or VEGF-A-positive cells in the stroma was observed (data not shown). However, both the number of CD105-positive and the number of CD31-positive vessels in the stroma associated significantly with expression of *VEGF-A* (mRNA) within the epithelial cell clusters [*P*=0.013 (Figure 3B) and *P*=0.005 (Figure 3C), respectively].


CD105
CD31
A1
B2
A
B
bFGF
VEGF
D1
C
D
VEGF RISH
Control
E
F

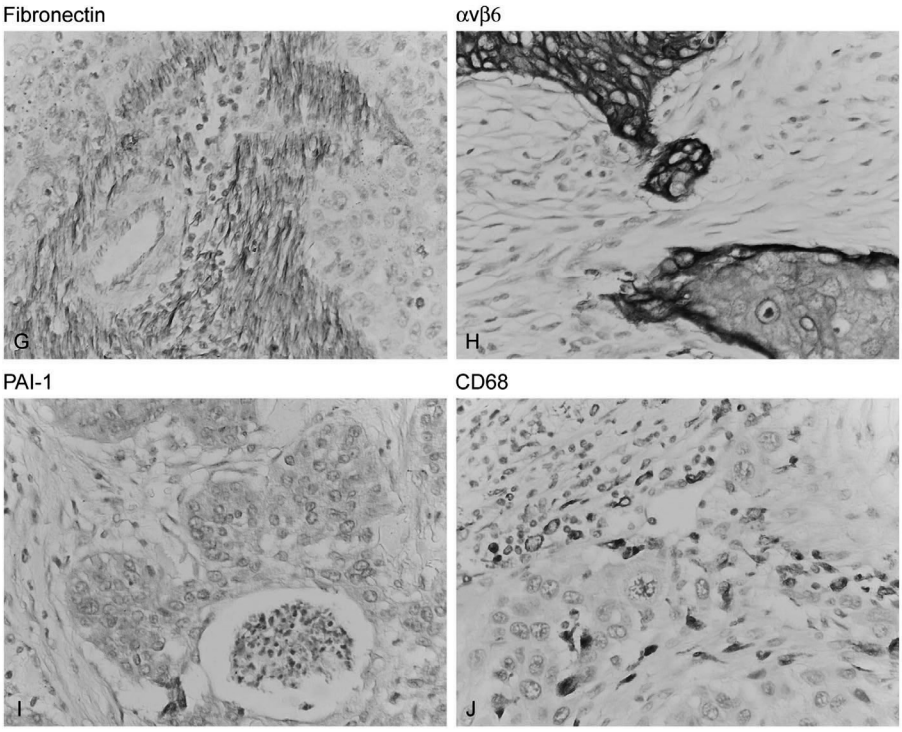


Figure 1. Expression and location of CD105- and CD31- positive vessels, bFGF, VEGF-A fibronectin, *αvβ6*, PAI-1 and CD68-positive cells.

Expression and location of CD105- and CD31- positive vessels, bFGF and VEGF-A were determined using immunohistochemistry as well as RNA-*in situ* hybridization (RISH) as described in Materials and methods section (magnification, x125). (**A**) CD105 vascular staining, (**A1**) Detail (magnification, x400) of vessels present in the tumor stroma as well as in the epithelial cell clusters. (**B**) CD31 vascular staining, (**B1**) detail (magnification, x400) of vessels present in the tumor stroma as well as in the epithelial cell clusters. (**C**) bFGF, positive staining of the border of the epithelial cell clusters and cells in the stromal compartment. (**D**) VEGF-A immunohistochemical staining with increased positive staining of the borders of the epithelial cell clusters, (**D1**) detail (magnification, x400) of VEGF-A positive stromal cells. (**E**) *VEGF-A* RISH with weak cytoplasmic staining of the epithelial cell clusters. (**F**) Negative (sense) control of *VEGF-A* RISH. Expression and location of fibronectin, *αvβ6*, PAI-1 and CD68-positive cells (magnification, x400). (**G**) fibronectin, positive staining (>75%) of the stromal compartment. (**H**) *αvβ6*, positive staining (strong intensity; of the border) of the epithelial cell clusters. (**I**) PAI-1, positive staining of the epithelial cell clusters. (**J**) Positive staining of CD68-positive cells in the stromal compartment and in the epithelial cell clusters. *See page 194-195 for color figure.*

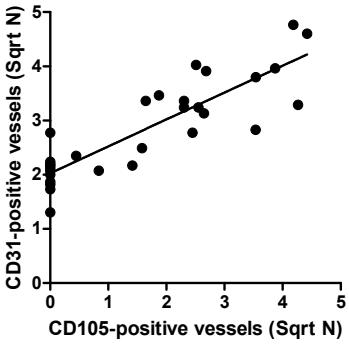


Figure 2. Correlation between CD105- and CD31-positive vessels.
Number of stromal CD105- and CD31-positive vessels was determined using immunohistochemistry as described in Materials and methods section (Pearson, r^2=0.747, P<0.001).

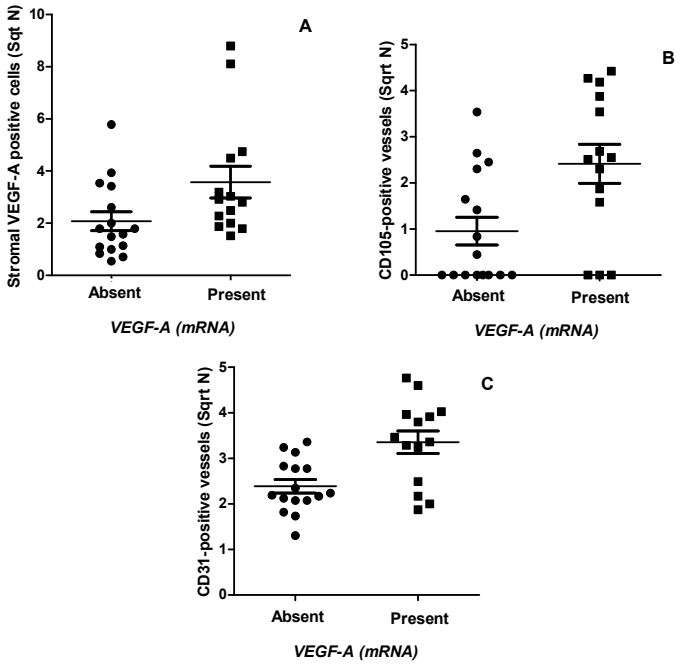


Figure 3. Association between *VEGF-A* (mRNA) expression measured in the epithelial cell clusters and the number of VEGF-A positive cells and blood vessels.
(**A**) Correlation between *VEGF-A* (mRNA) expression measured in the epithelial cell clusters and the number of VEGF-A positive cells (Mann-Whitney *U*-test, *P*=0.048) in tumor stromal compartment; (**B**) Correlation between *VEGF-A* (mRNA) expression measured in the epithelial cell clusters and the number of stromal CD105-positive vessels (Mann-Whitney *U*-test, *P*=0.013); (**C**) Correlation between *VEGF-A* (mRNA) expression measured in the epithelial cell clusters and the number of stromal CD31-positive vessels (Mann-Whitney *U*-test, *P*=0.005).

Association between blood vessels and tumor-associated macrophages

As angiogenic factors are also produced by tumor-associated macrophages (TAM), we have enumerated the number of CD68-positive cells[25] and correlated their number with the number of CD105- and CD31-positive stromal vessels. The presence of CD105-positive vessels in the tumor was significantly associated with total (stroma and epithelial cell clusters) number of CD68-positive cells (P=0.004; Figure 4A). However, the number of CD105-positive vessels in the stroma was not significantly correlated with the total number of CD68-positive cells. In addition, there was a significant correlation between the number of CD31-positive vessels in the stroma and the total number of CD68-positive cells (r^2=0.150, P=0.034; Figure 4B).

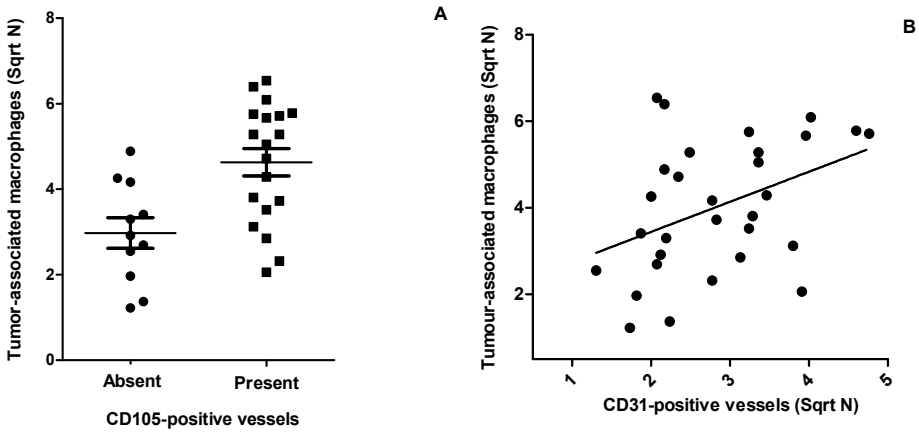


Figure 4. Association between tumor-associated macrophages and blood vessels.
(**A**) Association between the number of total (stroma and epithelial cell clusters) tumor-associated macrophages and the presence of CD105-positive vessels (Mann-Whitney U-test, P=0.004); (**B**) Correlation between the total number of tumor-associated macrophages and the number of stromal CD31-positive vessels (Pearson, r^2=0.150, P=0.034).

Association between blood vessels and TGF-β_1 target gene products PAI-1 and fibronectin and the TGF-β_1 activator $\alpha v\beta 6$

To explore the role of TGF-β_1 in the generation of CD105-positive vessels both the presence of CD105-positive blood vessels in the epithelial cell clusters and the number of blood vessels in the stroma were associated with the presence of PAI-1 in the epithelial cell clusters[17] and the amount of fibronectin in the stroma,[18] both

target genes of TGF-β_1. Neither the presence of CD105-positive vessels within the epithelial cell clusters nor the number of stromal CD105-positive vessels associated significantly with PAI-1 expression (data not shown). However, an association between the number of stromal CD31-positive vessels and high levels of fibronectin (>75%) was found (P=0.038; data not shown). Furthermore, a significant association between the number of stromal CD31-positive vessels and moderate or strong staining intensity (vs no or weak staining intensity at the epithelial-stromal interface) of $\alpha v\beta 6$ was observed (P=0.006; Figure 5B). Although a difference between the number of stromal CD105-positive vessels and moderate or strong staining intensity (vs no or weak staining intensity at the epithelial-stromal interface) of $\alpha v\beta 6$ was observed (Figure 5A), this difference was not significant (P=0.053). In addition, an association between the total number of TAM and $\alpha v\beta 6$ staining intensity was found (P=0.002; Figure 5C).

Association between blood vessels and clinicopathological parameters

Finally, we have associated both the presence of CD105-positive blood vessels within the epithelial cell clusters and the number of stromal CD105- or CD31-positive blood vessels with the FIGO stage, lymph node metastasis, tumor size, infiltration depth, vascular space involvement, parametrial invasion, human papilloma virus (HPV) status and histology. No significant associations were found between the majority of these parameters and the presence of CD105-positive blood vessels within the epithelial cell clusters or the number of stromal blood vessels. Only the presence of CD31-positive vessels in the epithelial cell clusters associated significantly with vascular space involvement (Fisher's exact test, P=0.021).

The number of both CD105- and CD31-positive stromal vessels was significantly associated with the presence of positive lymph nodes (P=0.005 and P=0.011, respectively).

In addition, the number of CD105-positive stromal vessels was significantly associated with the number of positive lymph nodes (P<0.001; Figure 6A). Finally, the presence of CD105-positive vessels within the epithelial cell clusters showed a negative relationship with disease-free survival (Figure 6C; P=0.007). Sample size was too small to calculate whether or not this is an independent prognostic variable.

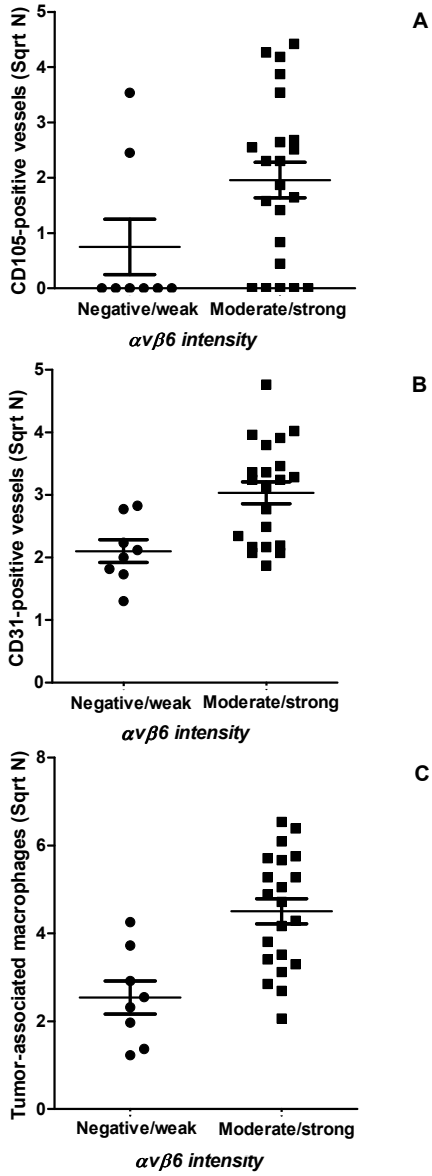


Figure 5. Association between *αvβ*6 expression and CD31-positive vessels and tumor-associated macrophages.

(**A**) Association between the presence of moderate/strong *αvβ*6 intensity and the number of stromal CD105-positive vessels (Mann-Whitney *U*-test, *P*=0.053); (**B**) Association between the presence of moderate/strong *αvβ*6 intensity and the number of stromal CD31-positive vessels (Mann-Whitney *U*-test, *P*=0.006); (**C**) Association between the presence of moderate/strong *αvβ*6 intensity and the total number of tumor-associated macrophages (Mann-Whitney *U*-test, *P*=0.002).

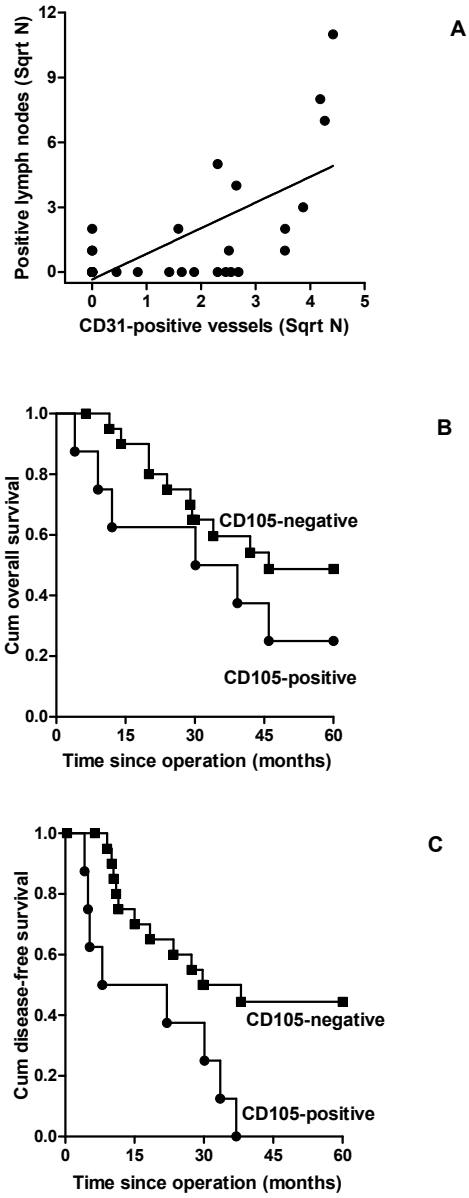


Figure 6. Association between CD105-positive vessels, the number of positive lymph nodes and survival. (**A**) Correlation between the number of stromal CD105 positive vessels and the number of positive lymph nodes (r^2=0.615; P<0.001); (**B**) Overall survival (Kaplan-Meier, Log-rank 1.55, P=0.214); (**C**) Disease-free survival (Kaplan-Meier, log-rank 7.28, P=0.007), both stratified by presence of CD105-positive vessels within the epithelial cell clusters of cervical carcinoma. CD105 absent in epithelial cell clusters is represented as the curve with a circle, CD105 present in epithelial cell clusters is represented by a square.

DISCUSSION

In this study, we have investigated the role of endoglin (CD105), a regulator of TGF-β_1 signaling on endothelial cells, bFGF and VEGF-A expression in 30 cervical carcinoma specimens. Most vessels were detected in the stroma as could be expected, as a pre-existing vascular network is necessary for developing new vessels.[26] Only one recent study has reported on the expression of CD105 in cervical cancer.[27] In agreement with this study, we also observed a positive correlation between the number of (newly formed) CD105- and (total number) CD31-positive vessels. In addition, we observed both CD105- and CD31-positive vessels within the epithelial cancer cell clusters. As blood vessels originate from the vasculature in the stroma, we assume that these vessels are embedded in a thin layer of stroma and surrounded by epithelial cancer cells.

Subsequently, we have assessed the association between the number of CD105-vessels and bFGF- and VEGF-A expressing cells. We did not observe an association between bFGF-expressing cells with the number of CD105 (or CD31)-positive vessels. An increase in *bFGF* mRNA expression has been reported during cervical tumor development,[28, 29] whereas a decrease in *bFGF* mRNA expression was reported in the study of Soufla *et al*.[30] To our knowledge, our study is the first study on bFGF-expressing cells in cervical carcinoma at the protein level using immunohistochemistry. Our study confirmed the absence of a statistical significant association between *bFGF* and *VEGF-A* expression in cervical cancer (data not shown) as earlier shown by Van Trappen *et al* using PCR-analysis.[29]

A negative association between VEGF-A protein expression and microvessel density, was reported by Tjalma *et al* in a study comprising 152 cervical carcinoma patients.[31] In our study, we did not find an association between either the number of CD105 (or CD31)-positive vessels (microvessel density) and VEGF-A protein expression. In contrast to the study of Tjalma *et al*, in our study we have also determined the presence of *VEGF-A* mRNA expressing cells. We were able to show a positive association between expression of *VEGF-A* mRNA and both CD105 expression and CD31 expression, suggesting that expression of *VEGF-A* mRNA is positively associated with microvessel density. The associations remained significant even

after a Bonferroni's correction for multiple testing by a factor 3. The presence of *VEGF-A* mRNA only detectable in the centre of the epithelial cell clusters, suggests that this is caused by hypoxia.[32] Although VEGF-A is considered to be one of the most important factors involved in angiogenesis,[26] surprisingly VEGF-A protein present at the epithelial-stromal interface did not correlate with the number of CD105 (or CD31)-positive vessels. This might be explained by consumption of VEGF-A protein by target cells.

The role of TAM in pressing an angiogenic switch has been highlighted by Lin and Pollard,[33] suggesting that TAM significantly contribute to angiogenesis. TAM are known to contain many proangiogenic factors, such as VEGF,[34] TNF-α,[35] CXCL-8,[36] bFGF,[37] and proteases such as MMP-2 and MMP-9.[38] These MMP are able to free pro-angiogenic cytokines, such as TGF-β_1 and VEGF-A, from the matrix. Indeed, we could show a positive association between the presence of CD105-positive vessels in the tumor, the stromal number of CD31-positive vessels and the total number of TAM. In contrast, an earlier study by Davidson *et al*, using CD31 as a vessel marker did not find this association.[39] Our results suggest that the presence of TAM is associated with neo-angiogenesis. However, there was no significant relationship between the number of VEGF-A-positive cells in stroma or VEGF-A protein in the epithelial cancer cell clusters and the number of TAM. This suggests that other factors in addition to VEGF-A contribute to angiogenesis. These factors may be induced by cytokines from TAM or liberated from the extracellular matrix by MMP derived from TAM. In addition, we did not observe a significant association between MMP-2 expressed at the border between the epithelial cancer cells and VEGF-A protein.[21]

TGF-β_1 plays an important role in angiogenesis, by promoting proliferation and migration of endothelial cells or promoting vessel maturation.[13] Furthermore, TGF-β_1 is known to induce VEGF expression.[15] We did not observe an association between the presence or the number of CD105-positive vessels and PAI-1, fibronectin (surrogate markers of TGF-β_1 activity), $\alpha v\beta 6$ and MMP-2 (activators of TGF-β_1) expression. However, the total number of (CD31-positive) vessels correlated with the expression of $\alpha v\beta 6$. In addition, $\alpha v\beta 6$ correlated significantly with the amount of fibronectin (P=0.037, data not shown), which is one of the factors that supports blood vessel growth.[16] Interestingly, $\alpha v\beta 6$ is also known to upregulate MMP-9 expression and thus

may also contribute to the release of VEGF-A from the extracellular matrix.[40]

On the basis of all our findings, we suggest that TGF-β_1 also plays a central role in the progression of cervical carcinoma (Figure 7). Latent TGF-β_1 is produced amongst others by cervical cancer cells, secreted and stored in the extracellular matrix. This TGF-β_1 is then processed by either the epithelial cell specific integrin $\alpha v\beta 6$[16, 41] or by MMP, especially active MMP-2[21] in complex with MMP-14 and tissue inhibitor of metallo proteinase (TIMP)-2 on the cell membrane of cervical cancer cells at the epithelial cell-stroma border. Active TGF-β_1 can induce VEGF in the epithelial cancer cells and differentiates fibroblasts surrounding the epithelial cancer cells into myofibroblasts. Furthermore, active TGF-β_1, depending on the local concentration, either promotes endothelial proliferation and migration or promotes cytostasis and vessel maturation. In addition, active TGF-β_1 acts as an immunosuppressor by blocking the activity of the inflammatory cells and inducing FoxP3-positive regulatory cells[42] and T_H17 cells.[43]

The prognostic value of CD105 and CD31 has been assessed earlier in breast, colon and ovarian carcinomas.[44-47] High expression of CD105 correlated with increased risk of metastasis in lymph node-positive breast carcinoma patients.[45] In this latter study, CD105 was shown to have an improved predictive value compared with CD31. In agreement with this study, in cervical carcinoma we observed an association between the number of CD105-positive vessels and the number of positive lymph nodes. With respect to survival, only the presence of newly formed vessels (CD105) within the epithelial cell clusters was correlated with a poor disease-free survival.

Our study suggests that the presence of CD105-positive vessels in the epithelial cell clusters may be of use as a (poor) prognostic factor in cervical carcinoma. This is important as CD105 has been proposed as a marker of tumor vasculature and a potential target for therapy of cervical carcinoma.[11]

Acknowledgements

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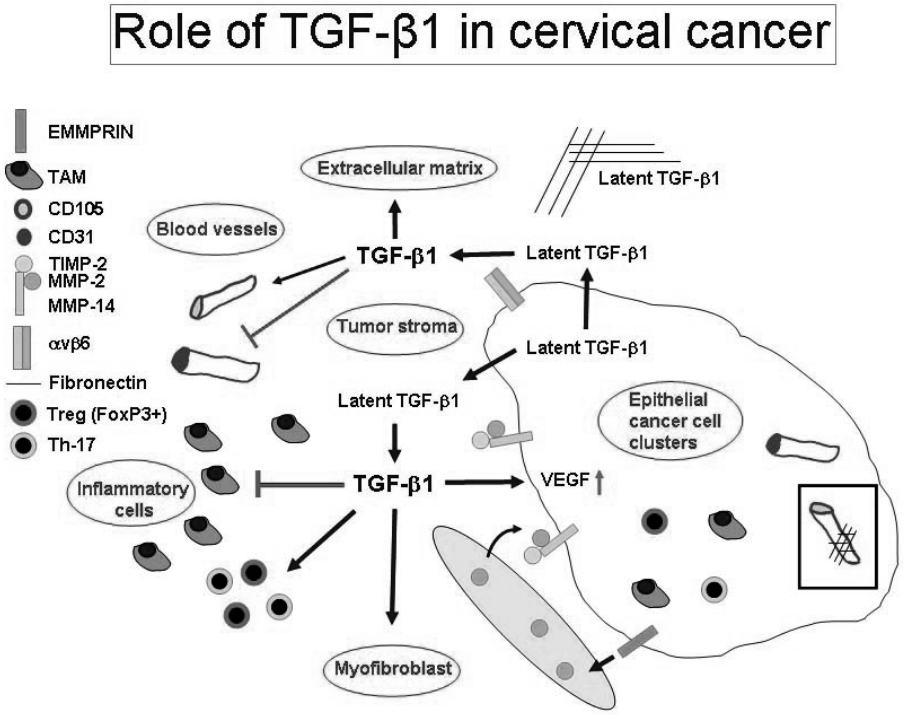

Figure 7 Role of TGF-β_1 in cervical cancer.
Cervical tumors are composed of malignant epithelial cells, tumor stroma, comprising the tumor vasculature extra cellular matrix and (myo)fibroblasts, and an inflammatory infiltrate. Latent TGF-β_1 is produced amongst others by cervical cancer cells, secreted and stored in the extracellular matrix. TGF-β_1 can be activated by the epithelial cell specific integrin $\alpha v\beta 6$ or by matrix metalloproteinases (MMP), especially active MMP-2 in complex with MMP-14 and TIMP-2 on the cell membrane of cervical cancer cells at the epithelial cell-stroma border. Active TGF-β_1 induces VEGF in the epithelial cancer cells and differentiates fibroblasts into myofibroblasts. Depending on the local concentration, active TGF-β_1, promotes endothelial proliferation and migration or promotes cytostasis and vessel maturation. Active TGF-β_1 acts as an immunosuppressor by blocking the activity of the inflammatory cells and inducing FoxP3-positive regulatory cells and T_H17 cells. *See page 196 for color figure.*

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CHAPTER 6

Endothelium specific matrilysin (MMP-7) expression in human cancers

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ABSTRACT

Overexpression of matrilysin (MMP-7) is predominantly associated with epithelial (pre)malignant cells. In the present study MMP-7 expression is also found in endothelial cells in various human cancer types. Endothelial MMP-7 was associated with CD34 and/or CD105 expression. These immunohistochemical data were confirmed by RT-PCR on VEGF-stimulated endothelial cells. In addition, MMP-7 was also identified in sprouting endothelial cells *in vitro*. The potential clinical relevance of endothelial MMP-7 was assessed for cervical cancer patients by evaluating the association with overall survival. In contrast to MMP-7 in malignant epithelial cells, MMP-7 expression in endothelial cells showed a significant association with poor survival (log-rank 5.12, P=0.02, n=30). Our data suggest that MMP-7 is involved in tumor angiogenesis, thereby contributing to malignant growth and hence associated with decreased survival.

INTRODUCTION

Matrilysin (matrix metalloproteinase-7, MMP-7) has long been regarded as the MMP exclusively produced by epithelial cells. Up-regulation of epithelial MMP-7 in early stage tumors is a consequence of mutations in the Wnt-signaling pathway.[1] In later tumor stages, hypoxia contributes to the induction of MMP-7 expression.[2] Only few non-epithelial cell types have been described to express MMP-7 *in vivo*. The first indication for MMP-7 production by endothelial cells in tumor tissue came from Nagashima *et al*,[3] but this important finding has never been confirmed. We evaluated the presence and the role of MMP-7 in endothelial cells in cancer. First, we investigated MMP-7 expression in endothelial cells within various tumor types in relation to co-expression with pan-endothelial marker CD34 and proliferating endothelium associated marker CD105.[4] Secondly, using real time (RT)-PCR, we determined whether VEGF, the classical initiator of angiogenesis, was able to induce MMP-7 expression in sprouting endothelial cells *in vitro*. In addition, we determined the effect of an MMP inhibitor on sprouting endothelial cells, in which MMP-7, either directly or indirectly, could be involved. Finally, to evaluate a potential clinical implication, we assessed the association between MMP-7 expression in tumor endothelial cells and survival of cervical cancer patients.

MATERIAL AND METHODS

Patient material

A total of 254 patients with untreated primary cervical carcinoma underwent a radical hysterectomy type III with lymphadenectomy between 1985 and 1995. Tissue samples were fixed in 10% formalin and embedded in paraffin. Samples from 30 patients were included in this study: mean age 45 years (range 29-72); FIGO stage: (20), IIA (8), IIB/IIIB (2); lymph node metastases: No (16), Yes (14); distant metastases: No (13), Yes (17); tumor size: <40 mm (16), ≥40 mm (12); infiltration depth <15 mm (15), ≥15 mm (9); vascular space involvement: No (10), Yes (20); parametrial invasion: No (20), Yes (10); HPV status: 16/18 (19), other types (6); tumor classification: squamous cell carcinoma (25), adenocarcinoma (1), adenosquamous carcinoma

(4). In addition, formalin-fixed, paraffin-embedded tissue blocks from patients with breast (n=5), stomach (n=5), colon (n=5), and prostate cancer (n=5) were used. Human samples were used according to the guidelines of the ethical committee of the Leiden University Medical Center.

Cells, chemicals and antibodies

Human umbilical vein endothelial cells (HUVEC) were isolated.[5] Cells from passage 3 to 6 were grown in M199 medium (Invitrogen, Breda, The Netherlands) containing 20% heat inactivated Fetal Calf Serum (FCS) (Perbio Science, Etten-Leur, The Netherlands), 0.05 mg/ml heparin, 2 mM Glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomycin (Invitrogen) and 12.5 μg/ml Endothelial Cell Growth Supplement (Sigma-Aldrich Chemie, Steinham, Germany) in fibronectin coated culture flasks (0.05 mg/ml). Human recombinant $VEGF_{165}$ was from Calbiochem (Calbiochem, San Diego, CA). Antibodies: MMP-7 [clone 111433, IgG2B mouse monoclonal detecting pro and active MMP-7 (R&D systems, Abingdon, UK)], monoclonal antibody (MAb) directed against CD34 (Invitrogen), MAb CD105 (DAKO, Glostrup, Denmark).

Immunohistochemistry

Paraffin sections were stained as previously described.[6] Deparaffinized and rehydrated 4 μm sections were quenched for endogenous peroxidase with 0.3% H_2O_2 in methanol before overnight incubation with primary antibodies diluted in PBA [phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA)]. Biotinylated rabbit anti-mouse immunoglobulin followed by horseradish peroxidase (HRP)-streptavidin complex (both DAKO) were applied for 30 min each. Immune complexes were visualized using 0.05% diaminobenzidine (DAB, Sigma-Aldrich Chemie), containing 0.0038% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) resulting in brown precipitate or alternatively Nova Red (Vector Laboratories, Burlingame, CA) resulting in red staining, and counterstained with Mayer's hematoxylin. Citrate antigen retrieval was used when indicated by the manufacturer. Appropriate tissue sections were included as positive controls and incubation with PBA without primary antibodies served as negative controls. MMP-7 staining in tumor and endothelial cells was independently scored by 2 individuals, integrating the percentage of cells stained and the intensity

of staining in a total score ranging from 0 to 8.[6] A score from 0-4 was considered as low and 5-8 as high.

RNA isolation and real-time RT-PCR

Total RNA was isolated from HUVEC cultured on fibronectin, gelatin or collagen I coated 6 well plates with or without 100 ng VEGF per well, using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcriptase PCR was carried out in 1 μg RNA using random primers and a cDNA synthesis kit (Promega, Leiden, The Netherlands). MMP-7 expression was quantified using real-time quantitative PCR according to the TaqMan method (Applied Biosystems, Weiterstadt, Germany) with *GAPDH* as endogenous housekeeping gene.[7] Double-stranded MMP-7 cDNA was used as positive control.

Sprouting assay

Endothelial spheroids were prepared from HUVEC[8, 9] in complete M199 medium containing 0.1% carboxymethylcellulose (Sigma-Aldrich) and 20% FCS, and seeded into non-adherent round bottom 96 well plates. After 24 h the spheroids were embedded in 1 mg/ml collagen matrix (Vitrogen 100; Nutacon, Leimuiden, The Netherlands) containing 20% FCS and treated with or without 100 ng/ml VEGF in complete M199 medium. Sprout-formation started after 3 h. Following overnight incubation, the spheroids containing collagen matrix were fixed in formalin and embedded in paraffin for immunohistochemical analysis.

MMP-7 activity assay

MMP-7 activity in cell culture media was determined using a recently developed immunocapture-based activity assay.[10]

Immunoblot

Samples were analyzed on 15% SDS-PAGE under non-reducing conditions. Proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). Blots were washed with PBST [PBS containing 0.05% Tween-20 (Merck, Darmstadt, Germany)] 3 times for 5 min. Non-specific binding was blocked with 0.2 % gelatin in

PBST for 30 min at room temperature. MMP was detected by incubation with anti-MMP-7 antibody (IgG2B mouse monoclonal antibody, R&D), followed by biotinylated goat-anti-mouse antibodies and Streptavidin-HRP (both DAKO). Coloration was performed using DAB.

Statistical analysis

Spearman correlations between parameters, survival curves, log-rank analyses and multivariate Cox analyses were performed using the SPSS 10.0 software package (SPSS Inc., Chicago, IL). *P* values ≤0.05 were considered significant.

RESULTS

Representative stainings of MMP-7 in endothelial cells in respectively stomach, colon, breast, cervix, and prostate cancer tissue is shown in Figure 1A-E. The unexpected staining of MMP-7 in endothelial cells was confirmed using three other anti-MMP-7 antibodies with similar results [rabbit polyclonal RP1MMP-7 (Triple Point biologics, Forest Grove, OR), rabbit polyclonal anti-MMP-7 (Abgent, Oxfordshire, UK) and goat polyclonal anti-MMP-7 (R&D)], data not shown. The presence of MMP-7 expression in endothelial cells was associated with co-staining for CD34 and CD105 in sequential sections from the same tissue, suggesting that these cells were of neo-angiogenic origin (Figure 1). MMP-7 expression in endothelial cells was in general less intense than expression in epithelial cells. MMP-7 staining in endothelial cells did not correlate with the expression in epithelial cells in the same sections (r^2=0.069, *P*=0.716, n=50).

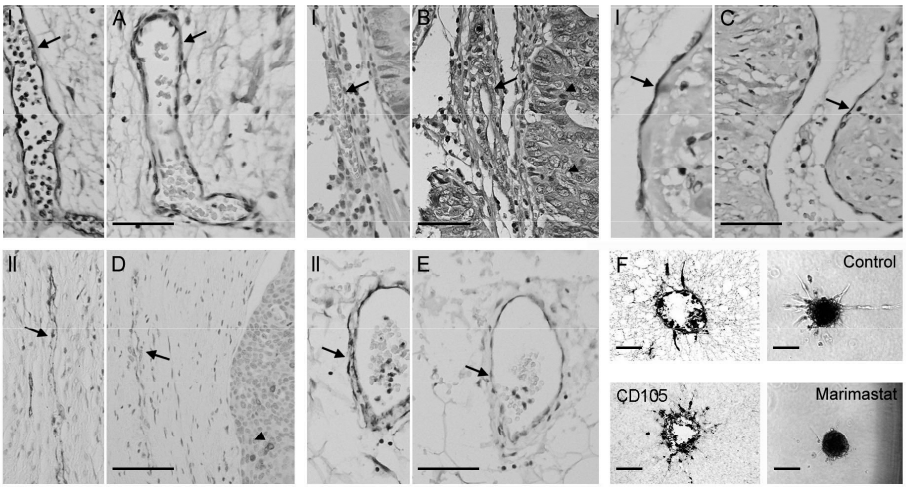

Figure 1. Immunohistochemical staining of endothelial cells for MMP-7, indicated by arrows in gastric cancer (**A**), colonic cancer (**B**), breast cancer (**C**), cervical cancer (**D**), prostate cancer (**E**), and in *in vitro* sprouting HUVEC cells (**F**). Inserts I and II indicate respectively CD34 and CD105 staining in sequential section from the same tissue. The inserts in (**F**) show VEGF-induced endothelial cell sprouting in control and Marimastat-treated HUVEC spheroids. Arrowheads indicate epithelial cell staining. Bars correspond with 100 μm in (**A**-**E**) and with 300 μm in (**F**). *See page 197 for color figure.*

MMP-7 expression is not expected to be present in endothelial cells. Therefore, we first demonstrated that endothelial cells are indeed able to express MMP-7 by evaluating *MMP-7* mRNA levels in HUVEC. Unstimulated HUVEC, cultured on fibronectin, did not express detectable quantities of MMP-7 mRNA (>40 cycles of RT-PCR). However, when grown on gelatin or collagen type I, HUVEC did express *MMP-7* mRNA, and VEGF stimulation up-regulated *MMP-7* mRNA expression more than 3-fold after 16 h (comparative C_t method: dC_t 11.84 versus 10.43 respectively). Longer periods of VEGF stimulation resulted in decreasing levels of *MMP-7* mRNA. Increased mRNA levels were reflected by the MMP-7 level in the conditioned medium. Serum-free medium from VEGF-stimulated HUVEC contained a similar level of MMP-7 as found in HT-29 colon cancer cell medium as quantified by a specific MMP-7 activity assay (24 versus 15 ng/ml respectively) and illustrated on the immunoblot in Figure 2 in medium and cell homogenates.

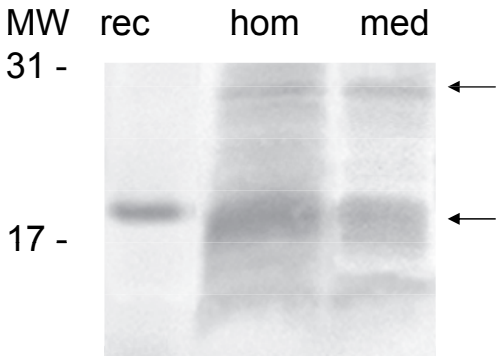

Figure 2. Immunoblot staining MMP-7 in endothelial cells and culture medium.
rec: recombinant active MMP-7; hom: homogenates of HUVEC grown on collagen type I and treated with 100 ng/ml VEGF; med: serum-free culture medium of HUVEC treated with VEGF. Arrows indicate presence of active (18 kDa) and pro-form (28 kDa) of MMP-7.

Subsequently, we used a 3-dimensional *in vitro* endothelial cell sprouting model to confirm our immunohistochemical and cell culture data. Figure 1F shows the presence of MMP-7 in VEGF-treated sprouting HUVEC. As expected, these cells stained also for CD105. Without VEGF stimulation HUVEC did not form sprouts and also stained less intense for MMP-7 and CD105 (data not shown). Addition of 1μg/ml Marimastat, a broad-range MMP inhibitor, prevented VEGF-mediated sprout formation completely (insert Figure 1F), suggesting the involvement of MMP activity in tumor angiogenesis. We evaluated the relation of MMP-7 expression in endothelial cells as well as cancer cells from the 30 cervical cancer patients with the clinicopathological parameters described in the Materials and methods section. None of these parameters were significantly correlated with MMP-7, except for the expression in endothelial cells with survival [low (score 0-4) versus high (score 5-8), log-rank 5.12, P=0.02, Figure 3A]. MMP-7 expression in tumor cells of these patients was not correlated with survival (log-rank 1.91, P=0.17, Figure 3B). In multivariate analysis against all the clinicopathological parameters all the univariate significant parameters, i.e. endothelial MMP-7 staining, FIGO stage, infiltration depth, and parametrial invasion lost their statistical significance due to the clinicopathological parameter distant metastasis (17 of 18 deceased patients had distant metastasis).

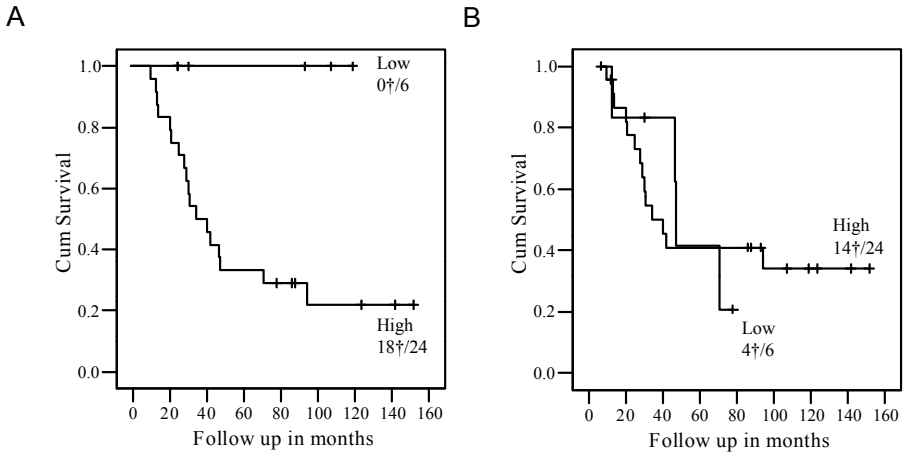

Figure 3. Kaplan-Meier survival curves for cervical cancer patients stratified for low (score 0-4) and high (score 5-8) MMP-7 immunohistochemical staining.
(**A**) MMP-7 in endothelial cells (log-rank 5.12, *P*=0.02). (**B**) MMP-7 in cancer cells (log-rank 1.91, *P*=0.17).

DISCUSSION

Previously, MMP-7 has been found in early adenoma epithelial cells, in malignant cells at the invasive front, and in colon-derived liver metastases.[11, 12] MMP-7 staining was also demonstrated in colonic endothelial cells adjacent to MMP-7 positive malignant epithelial cells, without detectable *MMP-7* mRNA levels in endothelial cells.[3] Our results show the presence of MMP-7 in angiogenic endothelial cells in various cancer types. Endothelial MMP-7 expression was not dependent on the proximity of malignant MMP-7 expressing epithelial cells, suggesting an endogenous endothelial origin. RT-PCR data indicated that endothelial cells under angiogenic conditions are indeed able to express *MMP-7*. Moreover, we have shown that under conditions closely resembling the *in vivo* neo-angiogenic process, MMP-7 up-regulation in endothelial cells coincides with sprout formation. Addition of Marimastat, a broad-range MMP inhibitor, prevented sprout formation completely without affecting cell viability. This suggests that endothelial cell-derived MMP are involved in neo-angiogenesis. From all MMP, especially MMP-7 could be of key importance in cancer, because it acts locally due to its cell membrane-adhering properties, secondly because it is able to activate other pro-MMP like MMP-2, -8 and -9, and thirdly

because of its capacity to release/cleave other important bioactive molecules.[13] The importance of MMP-7 in angiogenesis was underlined by the clinical data from our study, showing an association between endothelial MMP-7 expression and decreased survival in cervical cancer patients. Multivariate analysis did not reveal endothelial MMP-7 staining as an independent prognostic marker against clinicopathological parameters, but this was probably due to the strength of one of these parameters in this small population: 17 of the 18 deceased patients had a distant metastasis. Support for endothelial MMP-7 as a prognostic indicator was recently provided by a study in 156 renal cell cancer patients.[14]

In conclusion, MMP-7 expression was demonstrated in endothelial cells of various tumors and was associated with decreased survival in a cohort of cervical carcinoma patients. Considering the role of MMP-7 as local activator of proteinases and other biologically active proteins, specific inhibition of MMP-7 could contribute to inhibition of angiogenesis and anti-cancer therapy in general.

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CHAPTER 7

Versican expression is associated with tumor-infiltrating CD8-positive T-cells and infiltration depth in cervical cancer

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ABSTRACT

Cervical carcinoma is the second most frequent cancer type in women worldwide. Both inflammatory cells and stromal cells are important for tumor progression. Stromal cells produce growth factors and extracellular matrix and provide an adequate environment for angiogenesis. Versican, a member of the extracellular matrix, has been shown to have a role in tumor progression.

The aim of this study was to investigate versican expression, its association with tumor-infiltrating inflammatory cell subsets and with clinicopathological parameters in human cervical cancers. We have studied the expression of versican in 149 cervical cancers using immunohistochemistry and RNA-*in situ* hybridization. Versican was predominantly expressed in the stroma (myofibroblasts). Using quantitative real-time PCR, V0 was found to be the most prominent isoform. High stromal versican expression was significantly associated with a low number of tumor-infiltrating T cells (P=0.018) and particularly a low number of CD8-positive T cells (cytotoxic T cells; P=0.002). Stromal versican expression was significantly higher in patients with an infiltration depth ≥15 mm (P=0.004) and patients with parametrial invasion (P=0.044). Stromal versican expression was not associated with survival. Our results suggest that versican expression in the stromal compartment of cervical cancers results in reduced numbers of intraepithelial CD8-positive T cells and enhanced local invasion.

INTRODUCTION

Cervical carcinoma is the second most common cancer type in women world wide.[1] It is caused by a persistent infection with high-risk human papillomavirus (HPV) types. Next to malignant epithelial cells, this tumor consists of inflammatory cells and stromal cells. Both inflammatory and stromal cells are important for tumor progression.[2, 3] In cervical carcinoma there is a high amount of inflammatory infiltrate, and we have recently shown a correlation between a high number of intraepithelial regulatory T cells (Treg) and poor survival of cervical carcinoma patients.[4] Stromal cells produce growth factors and extracellular matrix (ECM) components and provide an adequate environment for angiogenesis. One of these ECM components is versican, which is a member of the large aggregating chondroitin sulphate proteoglycan (CSPG) family that has been implicated in tumor progression.[5] Versican is composed of a N-terminal globular G1 domain, a central glycosaminoglycan attachment region (GAG) and a C-terminal selectin-like G3 domain.[6] Four isoforms are generated by alternative splicing: V0 containing both GAGα and GAGβ domains, V1 containing the GAGα domain, V2 containing the GAGβ domain, and V3 consisting only of the globular domains.[7] Recently, a fourth isoform, V4, has been found in breast cancer.[8] The different isoforms are implicated in different biological processes. The V0 isoform is highly expressed during embryonic development,[9] whereas in adult human tissues the V1 isoform is most prevalent.[10] V0 and V1 are also the predominant isoforms found in cancer tissue.[11-14] Versican expression promotes tumor growth by destabilizing focal cell contacts, thus hampering cell adhesion and regulating angiogenesis.[10, 15] Indeed, increased levels of versican have been reported in different tumor types such as adenocarcinomas,[16-18] squamous cell carcinomas,[19, 20] sarcomas,[21] mesothelomas,[22] and malignant melanomas.[23] In addition, versican expression has been associated with tumor progression and decreased survival in various tumor types.[24-26] In cervical cancer, versican has been suggested to enhance tumor invasion and metastases formation.[20]

In this study, we have investigated the presence, isotype and location of versican in cervical carcinoma. We have determined the association between the presence of versican and the presence of different tumor-infiltrating inflammatory cell

subsets. In addition, we have investigated the association of versican with several clinicopathological parameters such as lymph node positivity, disease-free and overall survival.

MATERIALS AND METHODS

Tissue samples

A total of 149 patients treated with radical hysterectomy and bilateral pelvic lymph node dissection for uterine cervical cancer were included in this study. Patients did not receive preoperative radiotherapy or chemotherapy. Treatment occurred between 1985 and 1999. Tissues had been fixed routinely in 10% formalin and embedded in paraffin. All tissues were retrieved from the archives of the Department of Pathology, Leiden University Medical Center. Samples were used according to the guidelines of the ethical committee of the Leiden University Medical Center. Clinical parameters were stored in a prospective database (administered by the department of Gynecology) containing over 200 items per patient. The patient characteristics are shown in Table 1.

Immunohistochemistry

Immunohistochemical analysis was performed on 4 μm paraffin sections, mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinized, rehydrated and treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. Antigen retrieval was performed (0.01 M citrate, pH 6.0) and sections were rinsed in phosphate-buffered saline (PBS). Subsequently, sections were stained for versican (overnight) using a 1:200 dilution of anti-human versican monoclonal antibody (clone 2B1, IgG1; Seikagaku Corporation, Tokyo, Japan) in PBS containing 1% bovine serum albumin (BSA). The slides were incubated with Powervision-Poly-HRP-goat anti-mouse/rat/rabbit IgG (Immunogenic, Duiven, the Netherlands) and immune complexes were visualized with diaminobenzidine. CD68-positive cells, CD1a (Langerhans' cells)-positive intraepithelial cells or DC-LAMP-positive stromal dendritic cells were visualized as described previously.[27]

Table 1. Clinicopathological patient characteristics

Clinicopathological variable	Category	N* (%)	Association with high versican expression (*P*)
FIGO	IB1	59 (46)	0.831
	IB2/IIA	69 (54)	
Histology	Squamous	111 (77)	0.391
	Adeno/adenosquamous	34 (23)	
Lymph node metastasis	No	98 (66)	0.078
	Yes	50 (34)	
Largest tumor diameter	<40 mm	67 (49)	0.465
	≥40 mm	70 (51)	
Vasoinvasion	No	56 (40)	0.205
	Yes	84 (60)	
Infiltration depth	<15 mm	82 (58)	**0.004**
	≥15 mm	60 (42)	
Parametrial invasion	No	123 (83)	**0.044**
	Yes	25 (17)	
HPV type	HPV 16/18	101 (81)	0.963
	Other	23 (19)	
Recurrent disease	No	87 (60)	0.937
	Yes	59 (40)	

* N<149 because data were not reported in the patient's chart.
Bold values indicate *P*<0.05.

Fluorescent immunohistochemisty

A double staining was performed with smooth muscle actin (SMA; clone ASM-1, IgG2a, 1:300; Oncogene Research Product, Boston, MA) and mouse monoclonal anti-human versican (clone 2B1, IgG1, 1:200; Seikagaku Corporation) or mouse monoclonal anti-human cytokeratin (clone AE1/AE3, IgG1, 1:500; DAKO Netherlands bv, Heverlee, Belgium). Furthermore, a double staining with mouse monoclonal anti-human versican and mouse monoclonal anti-CD8 (clone 4B11, IgG2b, 1:400; Novocastra Laboratories, Newcastle upon Tyne, UK) was performed. Antigen retrieval with 0.01 M citrate pH= 6.0 was performed by boiling the slides for 10 min in a microwave.

The primary antibodies were diluted in 2% PBS/BSA in one mixture. The slides were incubated overnight with this mixture (100 μl per sample). The next day, the slides were washed with PBS. The slides were then incubated for 1 h with the secondary antibodies [100 μl per sample; goat-anti-mouse IgG2a ALEXA 546 (red), 1:200; goat-

anti-mouse IgG1 ALEXA 488 (green), 1:200, and goat-anti-mouse IgG2b ALEXA 647 (blue), 1:200]. All ALEXA antibodies were obtained from Invitrogen (Invitrogen, Breda, The Netherlands). After this, the slides were washed with PBS and then mounted with MOWIOL (a high quality anti-fade medium used for immunofluorescence).

RNA-*in situ* hybridization

Primers were chosen on the basis of the versican mRNA sequence (Gen Bank accession number GI_28144902): 5'-CAAGCATCCTGTCTCACGAA-3' (sense) and 5'-TTGGTATGCAGATGGGTTCA-3' (anti-sense). Probes were prepared and cervical carcinoma sections were stained for versican mRNA as previously described.[28] In short: 3 μm paraffin sections were pre-treated and hybridized with 100 ng/ml digoxigenin-labeled mRNA probe diluted in hybridization mixture containing 0.3 M NaCl and 0.03 M saline-sodium citrate (SSC). Hybridization was allowed for 16 h at 55°C in a humidified chamber. Slides were washed for 30 min in 50% formamide/2x SSC at 42°C, followed by 45 min in 0.1 x SSC with 20 mM β-mercaptoethanol at 50°C and for 30 min with 2 U/ml ribonuclease T1 (Roche Diagnostics, Mannheim, Germany) in 2x SSC, 1 mM EDTA at 37°C. RNA hybrids were detected using subsequently mouse anti-digoxigenin (1:2000; Sigma-Aldrich Chemie, Steinham, Germany), rabbit anti-mouse immunoglobulin (1:50; DAKO, Glostrup, Denmark) and mouse alkaline phosphatase anti-alkaline phosphatase (DAKO). A cervical cancer sample stained for TGF-β1 mRNA served as a positive control.

Evaluation of immunohistochemical staining and mRNA-*in situ* hybridization

Versican expression was scored as described by Ruiter *et al.*[29] Intensity was scored as none (0), mild (1), moderate (2) or intense (3) at low magnification (x100). Furthermore, the percentage of positive tumor cells was determined and divided into groups: 0% (0, absent), 1% to 5% (1, sporadic), 6% to 25% (2, local), 26% to 50% (3, occasional), 51% to 75% (4, majority) and 76% to 100% (5, large majority). The two parameters were combined, representing the sum of both the percentage and the staining intensity of the positive cells, which resulted in an overall score (0 or 2-8). The scores were combined into two groups: category 0 (score 0-5, low expression) and category 1 (score 6-8, high expression). Expression was scored by two independent

researchers without knowing the identity and clinical outcome of patients. The data on T-cell infiltration in this patient series were previously published.[4]

Quantitative real-time PCR

In all 2 μg of total RNA was transcribed to cDNA with Superscript II reverse transcriptase (Life Technologies, Grand Island, NY). Amplification reactions were performed with qPCR Core kit for SYBR Green (Eurogentec, Hampshire, UK) according to manufacturer's protocol. Fluorescent PCR analysis was performed using the BIO-RAD iCycler (BIO-RAD, Hercules, CA). The following PCR conditions were used: 10 min at 95˚C, followed by 45 cycles of 15 seconds at 95˚C and 1 min at the appropriate annealing temperature. The household gene *EEF1A1* was used for normalization of the expression data.[30] Primers were designed according to Zhao *et al*.[31] Relative quantification was performed using standard curves, followed by adjustment with the normalization factor, which was calculated using the Genorm program. The primer sequences are listed in Table 2.

Table 2. Primer sequences for qRT PCR

Gene		Sequence	Size (bp)
EEF1A1	Forward:	5'- CTGGCAAGGTCACCAAGTCT-3'	99
	Reverse:	5'- CCGTTCTTCCACCACTGATT-3'	
Versican V0	Forward:	5'-CCAGCAAGCACAAAATTTCA-3'	162
	Reverse:	5'-TGCACTGGATCTGTTTCTTCA-3'	
Versican V1	Forward:	5'-CCCAGTGTGGAGGTGGTCTAC-3'	124
	Reverse:	5'-CGCTCAAATCACTCATTCGACGTT-3'	
Versican V2	Forward:	5'-TCAGAGAAAATAAGACAGGACCTGATC-3'	135
	Reverse:	5'-CATACGTAGGAAGTTTCAGTAGGATAACA-3'	
Versican V3	Forward:	5'-CCCTCCCCCTGATAGCAGAT-3'	71
	Reverse:	5'-GGCACGGGGTTCATTTTGC-3'	

bp indicates base pair

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL). Data were processed using a chi-square test, Student's t-test, the Mann-Whitney *U*-test or the Fisher's exact test, depending on the number and distribution of the compared groups. $P<0.05$ was considered statistically significant.

RESULTS

Versican is predominantly expressed in the stroma

First, we investigated the presence and location of versican in 149 cervical cancer specimens using immunohistochemistry. Versican expression was predominantly observed in the stroma (Figure 1a-d). The expression of versican showed a heterogeneous staining pattern and was more pronounced at the invasive border in 127 out of 142 cervical cancer specimens. In 62 out of 149 specimens, versican expression was also found in the cervical carcinoma cells (Figure 1d, arrow). Tumors without versican expression were not observed. To quantify the versican expression, the scoring system of Ruiter *et al* was used.[29] Weak immunoreactivity (score 2-5) was observed in 93 cases, and strong immunoreactivity (score 6-8) was observed in 56 cases. As a control, the versican expression was determined in normal cervical tissue (Figure 1e). In this case, moderate expression of versican was found in the subepithelial tissue. As strong expression was found at the stromal-epithelial border, we determined whether tumor cell or (myo)fibroblast were associated with versican expression (Figure 2). For this purpose, immunofluorescent double staining with both anti-cytokeratin (tumor cells) and anti-SMA (myofibroblasts) and versican was performed. None of the tumor cells expressed in addition to keratin SMA (Figure 2a). The majority of versican expression was associated with myofibroblasts and only a few versican-positive tumor cells were identified (Figure 2b). Strong versican expression was observed around blood vessels in the tumor stroma.

To determine whether the tumor or the stromal cells in cervical carcinomas produce versican, RNA-*in situ* hybridization was performed using placental tissue as a positive control. Placental tissue, strongly positive according to the qRT-PCR results, showed a weak staining at the tip of the villi. Only a weak expression of versican was detected in the cervical cancer tissue. The expression of versican in cervical cancer tissue was predominantly present in the stromal compartment (Figure 1f).

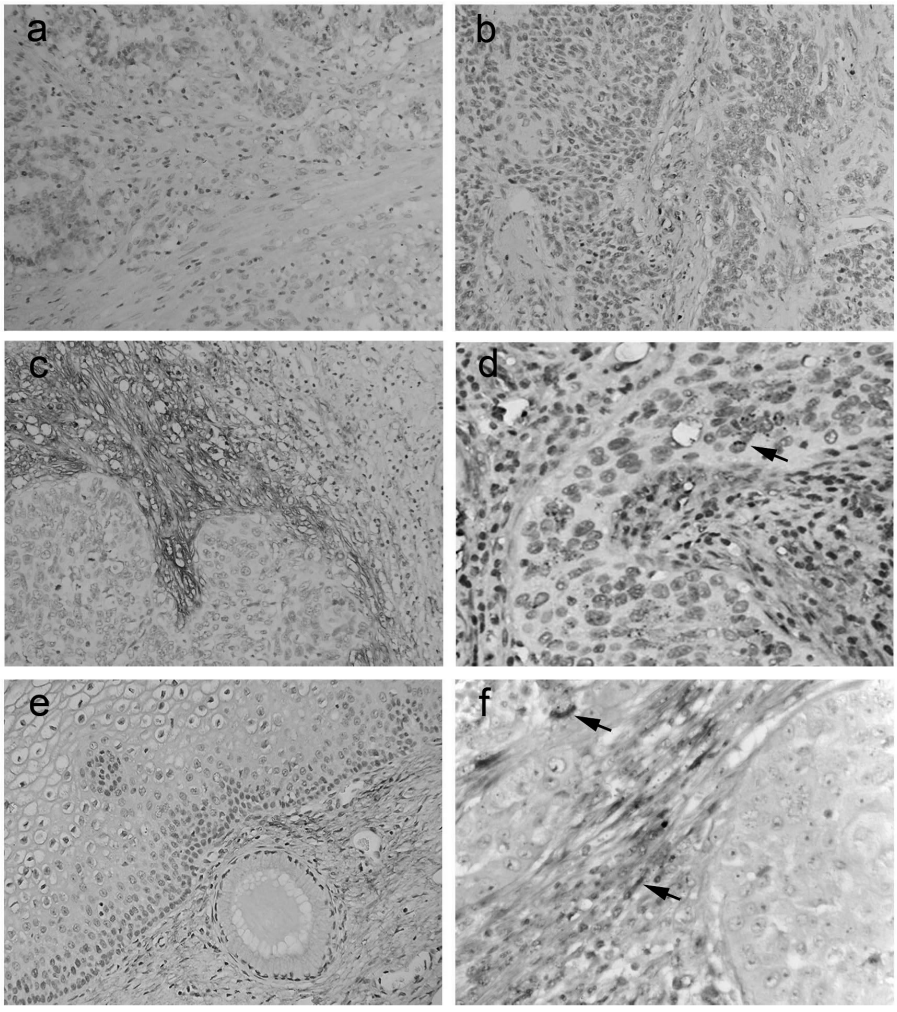

Figure 1. The expression and location of versican.
The expression and location of versican was determined using immunohistochemistry as well as RNA-*in situ* hybridization as described in Materials and methods (x250 magnification). (**a**) Cervical tumor, weak versican staining; (**b**) Cervical tumor, moderate stromal versican staining; (**c**) Cervical tumor, strong stromal versican staining; and (**d**) Cervical tumor, strong stromal versican staining (x400 magnification). The arrow indicates a positive tumor cell. (**e**) Subepithelial versican staining in normal cervical tissue and (**f**) RNA-*in situ* hybridization cervical tumor with weak cytoplasmic staining of stromal cell clusters (magnification, x400). Arrows indicate positive stromal cells. *See page 198 for color figure.*

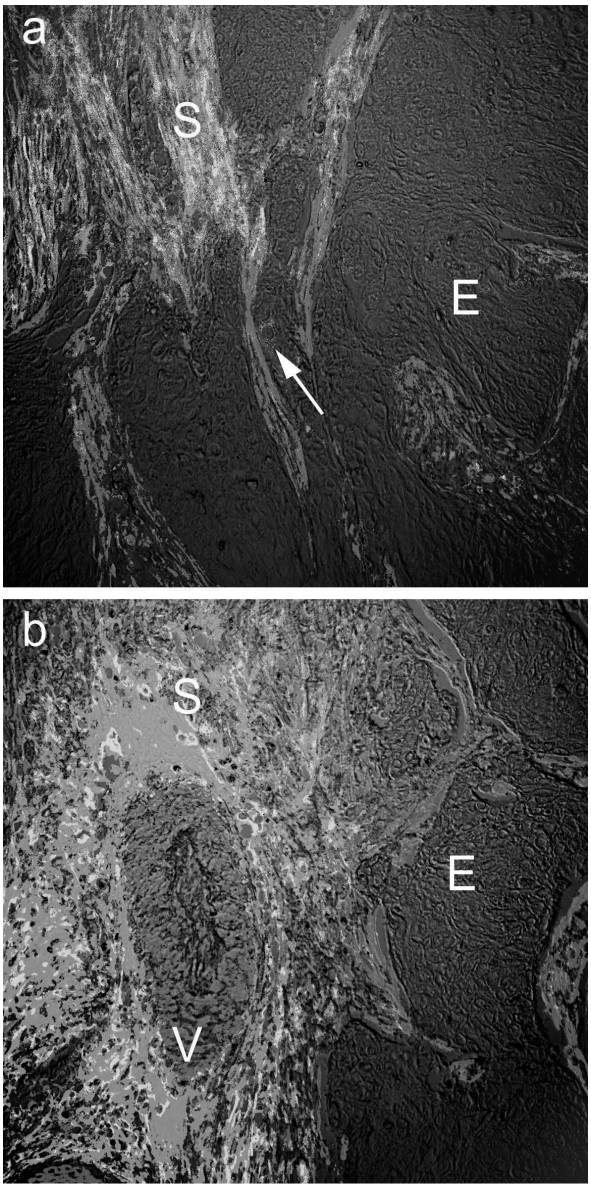

Figure 2. The expression and location of versican by stromal cells and tumor cells.
The expression and location of versican and smooth muscle actin (SMA) was determined using fluorescent immunohistochemistry as described in Materials and methods (magnification, x250). (**a**) Cervical tumor, strong stromal versican staining (green) and strong stromal SMA staining (red); the arrow indicates positive tumor cells, colocalization of versican and SMA in stromal cells (yellow). (**b**) Cervical tumor, strong stromal versican staining (green) and strong SMA staining of vessels (red). E, epithelial tumor cells; S, stroma; V, Vessel. *See page 199 for color figure.*

The V0 isoform is preferentially expressed in cervical cancer

Because versican can be expressed in different isoforms (V0, V1, V2 and V3), their expression was measured using qRT-PCR in 8 cervical cancer cell lines and 20 cervical carcinoma samples. Normal lung and placenta tissue served as a positive control. The expression level of each isoform was normalized to the household gene *EEF1A1*. From the cell lines investigated, CC10B expressed V0, V1 and V2, CSCC 7 expressed V1 and HeLa expressed V3. CaSki, CSCC1, CC8, CC11 and SiHa, did not express versican. To verify the mRNA expression pattern, versican protein expression was determined on cytospin preparations from CC10B and Caski. As expected, only CC10B showed prominent versican expression (data not shown). Previously, we have performed a cDNA microarray analysis on 32 tumor specimens.[32] All the cervical cancer samples examined in that study expressed versican (data not shown). In the present study, we have investigated the versican isoforms expressed in 20 tumor samples. V0 was found to be the most prominent isoform (Figure 3).

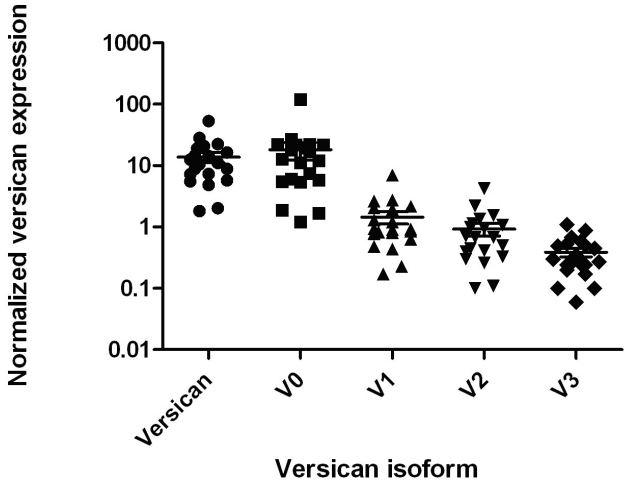

Figure 3. The expression of versican isoforms in cervical cancer.
Isoform expression was measured with qRT-PCR in 20 cervical specimens as described in Materials and methods. Versican expression was normalized against a household gene (*EEF1A1*).

High versican expression is associated with a decreased number of intraepithelial CD8-positive T-cells

As the composition of the ECM influences the migration of inflammatory cells,[33] we have also measured the association between inflammatory cells and versican expression. A statistically significant association was observed for tumors with a minor/moderate number of infiltrating inflammatory cells and tumors showing high versican expression (*P*=0.009; Table 3). Subsequently, we investigated whether this could be attributed to a particular chronic inflammatory cell subpopulation. Cell numbers were counted both in the stromal compartment and intraepithelial compartments. No statistically significant association was observed for tumors with a low (based on the median) number of tumor-associated macrophages (CD68-positive cells, both stromal and intraepithelial), Langerhans' cells (CD1a-positive cells; intraepithelial) or dendritic cells (DC-LAMP-positive cells; stromal) or natural killer (NK) cells and high versican expression. A statistically significant association between tumors with a low total number of intraepithelial CD3-positive T cells (*P*=0.018) and tumors with high versican expression was observed. No statistically significant association was observed for tumors with a low number of CD4-positive T cells, a low number of Treg cells or a low number of natural killer T (NKT)1 cells and tumors with high vesican expression. However, a strengthened statistically significant association between tumors with a low number of intraepithelial CD8-T cells (*P*=0.002) and tumors with high versican expression was observed. Using two-color confocal microscopy we could confirm this relationship (Figure 4). Indeed, tumors with a high versican expression showed a lower number of intraepithelial CD8-positive T cells (Figure 5, *P*< 0.018, Student's t-test)

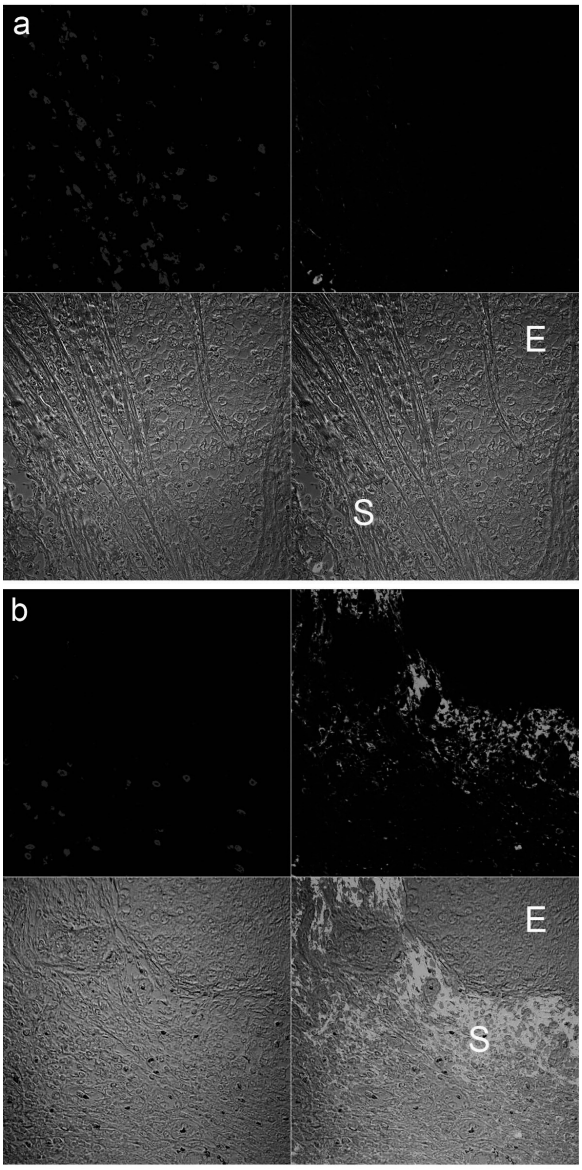

Figure 4. The expression of versican and location of CD8-positive T cells.
The expression of versican (green) and location of CD8-positive T cells was determined using fluorescent immunohistochemistry as described in Materials and methods (magnification, x250). The tumor is visualized as a phase-contrast Nomarski image (grey). (**a**) Cervical tumor, weak versican staining in the peripheral stromal area (green) was associated with a high number of intraepithelial CD8-positive T cells (blue); (**b**) Cervical tumor, strong versican staining in the peripheral stromal area (green) was associated with a low number of intraepithelial CD8-positive T cells (blue). E, epithelial tumor cells; S, stroma. *See page 200 for color figure.*

Table 3. Association between inflammatory cell subpopulations and versican expression

Cell population	Location	N	Median (mm^2)	Association with high versican expression (*P*)
Minor/moderate infiltration	IE†+stroma	101	NA*	**0.009**
Tumor-associated macrophages	stroma	33	156	1.000
Tumor-associated macrophages	IE	33	57	0.695
Dendritic cells	stroma	33	1	0.418
Langerhans'cells	IE	33	33	0.238
NK cells	IE	74	0	0.918
T cells (low numbers)	IE+stroma	74	180	**0.018**
CD4-positive T cells	IE	74	36	0.573
Regulatory T cells	stroma	70	24	0.508
Regulatory T cells	IE	70	4	0.060
NKT1 cells	IE	74	25	0.058
CD8-positive T cells (low numbers)	IE	74	94	**0.002**

* NA: not applicable.
† IE: intraepithelial.
Bold values indicate $P<0.05$.

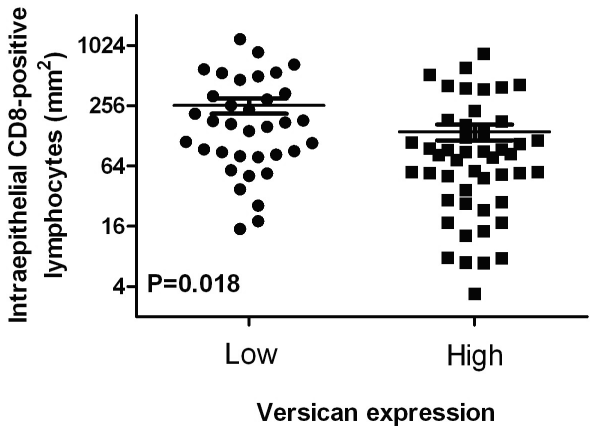

Figure 5. Association between versican expression and intraepithelial CD8-positive T cells. Versican and CD8 expression were determined using (fluorescent) immunohistochemistry as described in the Materials and methods. Immunohistochemical versican scores were combined into two groups: low versican expression (score 0-5), and high versican expression (score 6-8), as described in the Materials and methods. CD8-positive T cells were quantified as described in the Materials and methods and expressed as number per mm^2.

Versican expression is correlated with infiltration depth

As an association was found between high numbers of CD8-positive T cells and limited expression of versican, we determined whether this was also reflected in an association with clinical parameters. We have associated high or low versican expression with FIGO stage, lymph node metastasis, tumor size, infiltration depth, vascular space involvement, parametrial invasion, HPV status and histology (Table 1). Tumors with high versican expression were significantly associated with an infiltration depth ≥15 mm (*P*=0.004) and parametrial invasion (*P*=0.044). However, no statistically significant association between patients with tumors with high versican expression and patient survival was observed.

DISCUSSION

In the present study, we have investigated the expression of versican in cervical cancer and have correlated its expression with the presence of inflammatory cells and clinicopathological parameters. As observed by Kodama *et al*, versican is predominantly expressed in the stromal compartment, although occasionally tumor cells are also positive.[20] As previously shown for breast tumors, in cervical tumors also intense versican staining was observed in the peripheral area of the tumors.[34] Using immunofluorescent double staining, we have shown that versican expression was associated with myofibroblasts. We have demonstrated that the predominant versican isotype in cervical cancer is V0. Previously, V0 and V1 were reported to be the predominant isoforms in other epithelial cancer types such as breast and prostate cancer.[8, 11-14] The V1 isoform has been shown to enhance cell proliferation and to protect fibroblasts from apoptosis.[35] Increased levels of versican have been found in brain tumors, cervical carcinomas, melanomas, breast cancer, prostate cancer, colorectal cancer, lung cancer, pancreatic cancer, endometrial cancer, oral cancer and ovarian cancer.[12, 13, 16-18, 20, 23, 34, 36-39]

As the composition of the ECM influences the migration of inflammatory cells,[33] we have also measured the association between inflammatory cells and versican expression. Despite the reported production of versican isoforms V0 and V1 by human leukemic monocytes[40] and the reported increased synthesis of versican by

hypoxic macrophages, no significant statistical association was observed for stromal or intraepithelial CD68-positive cells in our study. Recently, in a rat experimental model, fragments of versican were shown to activate toll-like receptor (TLR)2-positive macrophages.[41] We could demonstrate TLR2-positive macrophages in both the stroma and the epithelial compartment (data not shown). However, no statistically significant association between versican expression and the presence of TLR2-positive macrophages was observed. In addition, we did not find an association between TLR2-positive macrophages and TNF-α expression (data not shown). However, a statistically significant correlation between tumors with a low number of intraepithelial T cells and tumors with high versican expression was shown. Versican is known to bind several cytokines and chemokines, such as XCL1 (Lymphotactin), CCL5 (RANTES), CCL20 (LARC), and CCL21 (SLC), all of which are potent T cell chemoattractants.[42] It is noteworthy that CCL5 expression has been associated with CD8-positive T-cell infiltration[43] and that mouse 6Ckine (CCL21 in humans) when transfected into a colon carcinoma cell line has both antitumor activity mediated by CD8-positive T cells and angiostatic effects.[44] This suggests that versican might act as a molecular sink, trapping CCL5 and CCL21, and offers an explanation for the reciprocal relationship between versican expression and the presence of CD8-positive T cells.

We also have associated expression of versican with relevant clinical parameters such as lymph node metastasis, tumor size, infiltration depth and vascular space involvement. Although for breast, cervical, prostate, and other tumor types, increased versican expression has been associated with relapse and poor clinical outcome,[12, 16, 17, 19, 20, 36, 39, 45, 46] we could not demonstrate this association in our cervical cancer cohort. To exclude that this was related to the scoring system, we have also scored our data according to the method described by Kodama *et al*,[20] (data not shown) but in this case also no statistically significant association was found. However, we did find a statistically significant association with an infiltration depth of ≥15 mm (P=0.004). As there is a significant association between infiltration depth and survival, we stratified infiltration depth according to versican expression. The results show that (low or high) versican expression does not affect survival of cervical cancer patients with an infiltration depth of <15 mm, whereas in cervical cancer patients with infiltration depth of ≥15 mm, low versican expression predicts a poorer survival.

However, because of the limited number of cases (n=60), the survival difference between patients with a low versican or a high versican expression in this subgroup of cervical cancer patients was not significant (data not shown).

In the studies of Kodama *et al*,[20, 45] the depth of stromal invasion (cervical cancer) and myometrial invasion (endometrial cancer) was also associated with versican expression, although in both studies only a trend (P=0.069 and P=0.052, respectively) was observed. However, experimental support for a role of versican in invasion was provided by functional studies demonstrating that versican is able to increase cancer cell motility[47] and to reduce the attachment of cancer cells to fibronectin-coated surfaces.[48]

In conclusion, our results suggest that modulation of the ECM composition in the peripheral stromal area of cervical cancers influences the migration of cytotoxic T cells and enhances local invasion of the tumor cells.

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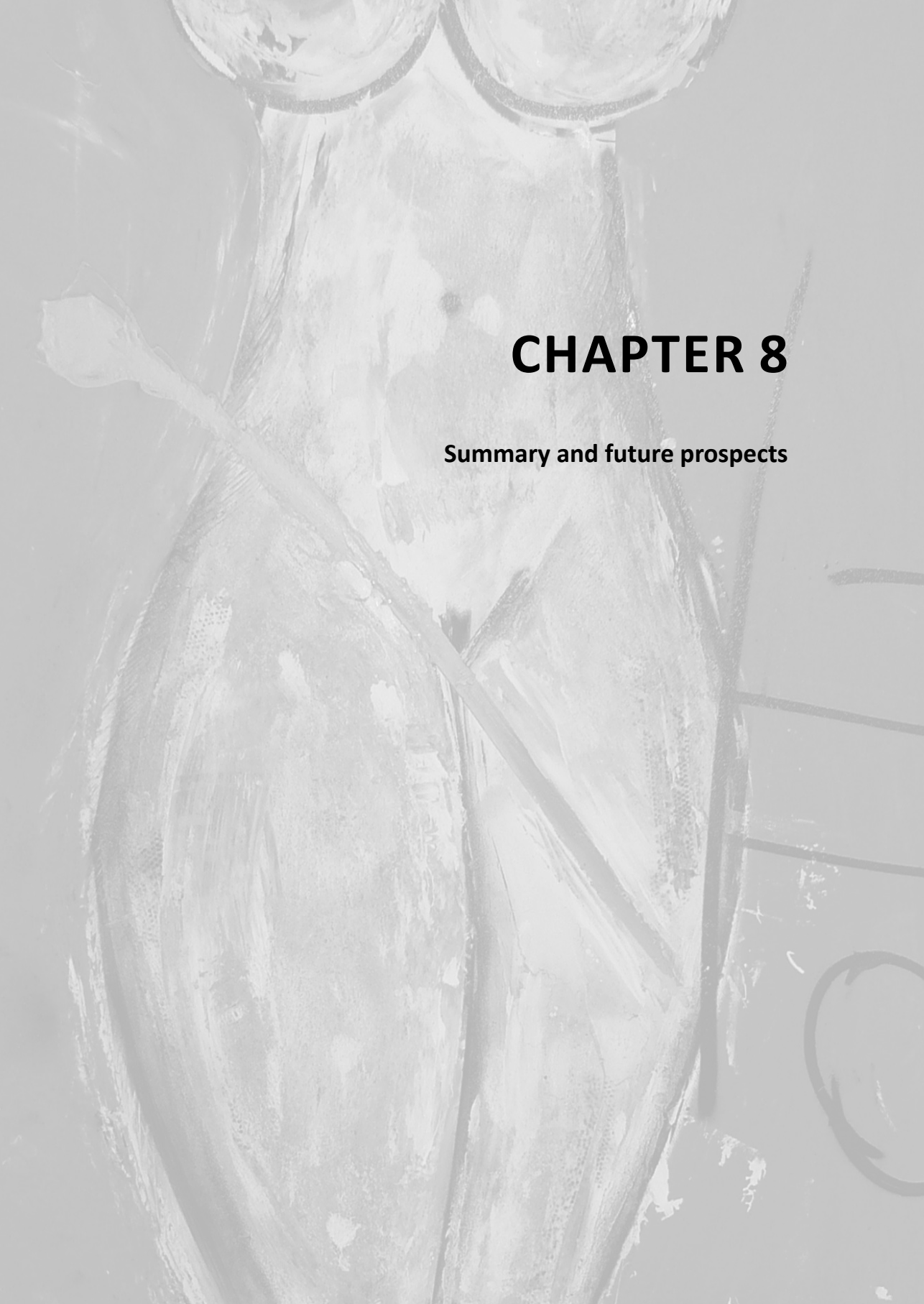

CHAPTER 8

Summary and future prospects

SUMMARY

Chapter 1 comprises a general introduction and the outline of this thesis. Cervical carcinoma is the third most frequent cancer type in women worldwide.[1] In addition to the properties of cervical cancer cells, both inflammatory cells and stromal cells are important for tumor progression.[2-4] Stromal cells produce growth factors and extracellular matrix and provide an adequate environment for angiogenesis.[5-7] Cervical carcinoma cells are able to influence this microenvironment by producing specific signaling molecules like cytokines, chemokines and proteinases.[8] The net result of produced signaling molecules differs, depending on the tumor stage. Proinflammatory cytokines are important in modifying the activity, differentiation, and migration of antigen-presenting cells and may influence the survival of cancer patients.[9, 10]

In **Chapter 2**, we determined whether *CCL2* mRNA expression is beneficial or detrimental for cervical cancer patients. For this purpose the association between the expression of *CCL2* by cervical tumor cells, the number of tumor-associated macrophages (TAM), and clinicopathological parameters such as recurrence, relapse-free survival, and overall patient survival was investigated. The *CCL2* mRNA expression level was quantified in cervical cancer samples from 93 untreated cervical cancer patients, using RNA-*in situ* hybridization (RISH) and verified by using real-time quantitative RT-PCR. The number of TAM was determined using immunohistochemistry. Furthermore, we investigated whether lack of *CCL2* expression was due to genetic alterations near the 17q11.2 (*CCL2* genomic) region. *CCL2* mRNA expression by cervical tumor cells was associated with the number of TAM ($P<0.001$). Lack of *CCL2* mRNA expression (15 samples; 16%) was associated with increased cumulative relapse-free survival (log rank test, $P=0.030$), increased cumulative overall survival (log-rank test, $P=0.024$), reduced local and distant recurrence, reduced vascular invasion, and smaller tumor size (<40 mm). The absence of *CCL2* mRNA expression corresponded with loss of heterozygosity (LOH) at 17q11.2 in five of six samples. The increased cumulative relapse-free survival and cumulative overall survival of cervical cancer patients lacking tumor cell-associated *CCL2* mRNA suggest that the TAM support tumor progression, presumably by promoting angiogenesis and production of growth factors.

In **Chapter 3**, we assessed whether *GM-CSF*, *TNF-α*, and *IL-12*, produced by cervical cancer cells, are important for the activity, differentiation and migration of antigen-presenting cells. In 90 patients with cervical carcinoma the number of monocytes/TAM, mature dendritic cells (DC), and Langerhans' cells (LHC) was determined using immunohistochemistry. A RISH technique was used to measure the expression level of *GM-CSF*, *TNF-α*, *IL-12p35*, and *IL-12p40*. TAM were detected intraepithelial as well as in the stroma of the tumor. LHC were predominantly detected intraepithelial and mature DC predominantly in the tumor stroma. The number of TAM correlated positively with the number of mature DC. The expression levels of *GM-CSF* and *TNF-α* correlated positively with the number of TAM and DC. *TNF-α* showed a negative correlation with the number of LHC. A significant correlation between the expression of functional IL-12 (*IL-12p40*) and stromal TAM was found. The expression of *GM-CSF*, *TNF-α*, and *IL-12p40* did not correlate significantly with disease-free survival. However, high *IL-12p40* expression was associated with a favorable cumulative overall survival. The results suggest that *GM-CSF* as well as *TNF-α*, produced by cervical carcinoma cells, may play a role in the differentiation of monocytes into mature DC. Furthermore, *TNF-α* may influence the migration of LHC from the tumor. Since IL-12p40 is both a subcomponent of IL-12 (composed of IL-12p40 and IL-12p35) and IL-23 (composed of IL-12p40 and IL-23p19),[11] in **Chapter 4** we elucidated the role of IL-12p40 in cervical cancer. For this purpose we measured the expression of *IL-23p19* mRNA in 90 cervical cancer specimens using RISH and compared its expression with *IL-12p40* and *IL-12p35* expression. Since IL-23 is a component of the IL-17 pathway,[12] we also studied IL-1 and IL-6 expression using immunohistochemistry since both cytokines are known for their induction of the IL-17/IL-23 pathway. *IL-12p40* expression was significantly associated with poor disease-specific survival (P=0.017). Also a high number of stromal IL-6 producing cells was shown to associate with poor disease-specific survival (P<0.001). However, the worst prognosis was associated with a subgroup of patients that displayed a high number of stromal IL-6 expressing cells and low *IL-12p40* expression (P<0.001). Furthermore, both a high number of stromal IL-6 expressing cells and a high number of stromal IL-6, plus *IL-12p40* expression showed to be independent clinicopathological parameters compared to lymph node metastasis, parametrial involvement and Sedlis score (P=0.009 and

P=0.022, respectively). Our results with IL-6 and *IL-12p40* are in accordance with the hypothesis that the IL-17/IL-23 pathway plays a suppressive role in cervical cancer.

In **Chapter 5**, we investigated the role of endoglin (CD105), a regulator of TGF-*β* signaling on endothelial cells[13], bFGF and VEGF-A. We measured the number and determined the location of both newly formed (CD105-positive) and overall number of (CD31-positive) blood vessels, and bFGF and VEGF-A production using immunohistochemistry in 30 cervical carcinoma specimens. *VEGF-A* mRNA expression was determined using RISH. CD105- and CD31-positive vessels and bFGF- and VEGF-A-positive cells were predominantly present in the stroma. The presence of CD105- and CD31-positive vessels in the stroma did neither correlate with the number of VEGF-A-positive cells nor the number of bFGF-positive cells. However, the number of CD105- and CD31-positive vessels was associated with the expression of *VEGF-A* mRNA in the epithelial cell clusters (*P*=0.013 and *P*=0.005, respectively). The presence of CD105-positive and CD31-positive vessels was associated with the expression of *αvβ*6 (a TGF-*β* activator; *P*<0.013 and *P*=0.006, respectively). Clinically, the number of CD105- positive vessels associated with the number of lymph node metastasis (*P*=0.001). Furthermore, the presence of CD105-positive vessels within the epithelial cell clusters associated with poor disease-free survival (*P*=0.007).

In **Chapter 6***,* the expression of MMP-7 was investigated in different cancer types. Over-expression of matrilysin (MMP-7) is predominantly associated with epithelial (pre)malignant cells.[14, 15] In addition to cancer cells, MMP-7 expression was found by immunohistochemistry in endothelial cells in various human cancer types. Endothelial MMP-7 was associated with CD34 and CD105 expression. The immunohistochemical data were confirmed by RT-PCR on VEGF-stimulated endothelial cells. Furthermore, MMP-7 was also identified in sprouting endothelial cells *in vitro*. The potential clinical relevance of endothelial MMP-7 was assessed for cervical cancer patients by evaluating the association with overall survival. In contrast to MMP-7 in malignant epithelial cells, MMP-7 expression in endothelial cells showed a significant association with poor survival (likelihood ratio 5.12, *P*=0.02, n=30). Our data suggest that MMP-7 is involved in tumor angiogenesis, thereby contributing to malignant growth and thus associated with decreased survival.

In **Chapter 7**, we investigated versican expression, its association with tumor-infiltrating inflammatory cell subsets and its association with clinicopathological parameters. Versican is a member of the extracellular matrix and has been put forward to play a role in tumor progression.[16-18] We studied the expression of versican in 149 cervical cancers using immunohistochemistry and RISH. Versican was predominantly expressed in the stroma (myofibroblasts). Using quantitative RT-PCR, V0 was found to be the most prominent isoform. High stromal versican expression was significantly associated with a low number of tumor-infiltrating T cells (P=0.018) and particularly a low number of $CD8^+$ T cells (cytotoxic T cells, P=0.002). Stromal versican expression was significantly higher in patients with an infiltration depth ≥ 15 mm (P=0.004) and in patients with parametrial invasion (P=0.044). Stromal versican expression was not associated with survival. Our results suggest that versican expression in the stromal compartment of cervical cancers results in reduced numbers of intraepithelial $CD8^+$ T cells and enhanced local invasion.

Growth and progression of cervical carcinoma is dependent on a complex interaction between cervical carcinoma cells and composition of the extracellular matrix.[19, 20] For local progression as well as metastasizing, the extracellular matrix needs to be rearranged creating space for tumor cells to expand and angiogenesis to secure supply of nutrients and oxygen and removal of waste products.[21, 22] The net result of all contributing factors will lead to either progression or degradation of cervical cancer.[23] In this thesis the role of contributing factors is investigated, e.g. cytokines, chemokines, inflammatory cells, the role of extracellular matrix and angiogenesis.

FUTURE PROSPECTS

Cervical cancer remains a health problem worldwide. In Western countries numbers are (slowly) declining the past decades[24] but in developing countries it still is often the most occurring malignancy among women.[25] Screening for cervical cancer is a very powerful tool to detect (pre)cancerous lesions and is a relatively cheap and easy technique to perform. Besides the risk for false-negative results,[26] there is also a risk for false-positive results, leading to the chance of over-treatment,[27] especially in the

group of women under 25 years of age.[28] Treatment of precancerous lesions and early stage cervical carcinoma gives excellent results but availability, accessibility and follow-up are not everywhere guaranteed.[29] Since the majority of patients suffering from cervical cancer are not participating in cervical cancer screening,[30-32] other methods are developed for early detection and/or prevention of developing cervical cancer. Self-collected vaginal specimens and visual inspection with acetic acid (VIA) on an outpatient base ("see and treat") with immediate treatment of suspicious lesions or HPV detection in cervical swabs are investigated as alternatives.[33-37] Still, for being an effective diagnostic tool, there must be a high coverage of screening (>80%), reproducibility and test properties must be sufficient and an effective treatment must be provided. HPV detection (self-collected or collected by a clinician) appears very promising[38] with sensitivity and specificity equal to or better than the classical Pap smears.[39]

However, these screening programs are all part of secondary prevention measurements. The costs of primary prevention by educating the population (the use of condoms, stop smoking) can be high and the effect can be difficult to measure.[40, 41] Since the development of vaccines to prevent infection with cancer-associated high-risk HPV type 16 and 18,[42] there is an alternative which most likely will lead to a reduction of cervical cancer and also other malignancies caused by HPV like vulvar, vaginal and anal cancer.[43, 44] Vaccination against HPV16/18 may be the first method to prevent a virally caused cancer. Especially when vaccines are developed which will target more high-risk HPV types, prevalence of HPV-associated malignancies will decline.[45]

Unfortunately, once sero-conversion has occurred and/or (pre)malignant cervical lesions have developed, prevention by prophylactic vaccination is not an option. After infection of the cervical epithelium with HPV, there is a limited opportunity for antigen presenting cells and Langerhans' cells within the epithelium to present antigens to the immune system since the viral particles do not cause lysis of the keratinocytes.[46] Without viremia, the chances for the systemic immune system to detect viral antigens leading to an adequate immune response are not optimal. Antibody responses to the capsid generally are weak and approximately half of the persons with a persistent high-risk HPV infection mount a measurable immune response.[42] To overcome these

problems, therapeutic vaccination is under investigation, using synthetic long peptide vaccines (SLP).[47] This immunotherapy is dependent on a cell-mediated immunity, needing antigen-presenting cells to present HPV antigens to T lymphocytes leading to a T-cell mediated immune response.[48] In patients with premalignant vulvar lesions induced by high-risk HPV, encouraging therapeutic results have been reported.[49-51] Also the use of modified dendritic cells as a vaccine is currently under investigation, for instance by activating them by double stranded RNA, resulting in increased antigen presentation and T cell stimulatory capacity.[52-54]

The effect of immunotherapy is not only dependent on antigen-presenting cells and T-cells, but also on the microenvironment surrounding these inflammatory cells (stromal cells as well as tumor cells).[55] Cytokines, chemokines and other components of this microenvironment like proteases are of great importance in activating antitumor immunity by, for instance, chemoattraction of antigen-presenting cells (CCL2),[56] activation of $CD8^+$ T lymphocytes (IL-17)[57] and differentiation of monocytes into mature dendritic cells (GM-CSF and TNF-α).[58, 59] For an optimal therapeutic vaccination in the future, a combination of a vaccine directed against multiple high-risk HPV types with adjuvant treatment boosting the local immune response may lead to a better outcome. Currently biological therapies are developed and investigated in the treatment against cervical cancer. Examples are Tocilizumab (RoActemra®), a humanized anti-IL-6 receptor antibody that could be of importance by inhibiting an IL-6 induced angiogenic switch.[60] Bevacizumab (Avastin®), a recombinant humanized anti-VEGF monoclonal antibody acting as an angiogenesis inhibitor. Bevacizumab is already used in phase II trials with limited activity in patients suffering from persistent or recurrent cervical carcinoma. Also Mapatumumab (HGS-ETR1), a human monoclonal antibody targeting the TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1), is currently investigated in a phase IB/II-study. Both Bevacizumab as Mapatumumab are used as an adjuvant therapy in combination with chemotherapy or radiotherapy and chemotherapy respectively, in advanced cervical carcinoma. From these reagents a synergistic effect is anticipated.[61-63]

Taking into account the differences in presence and amount of signaling molecules (cytokines and chemokines) as well as number and location of inflammatory cells, one can conclude that the host response elicited by neoplasms is complex and more

research is essential to find the best treatment. In the near future cervical carcinoma may become an even more rare disease due to a widely implemented prophylactic vaccination program, hopefully also available and affordable in developing countries. Furthermore, in these cases that cervical carcinoma does occur, maybe a personalized treatment can be offered by analyzing the primary tumor and tumor stroma characteristics in a biopsy of the tumor, leading to a specific (adjuvant) therapy in addition to surgery and/or radiotherapy whether or not in combination with chemotherapy or hyperthermia. Also other virally caused cancers can benefit from this knowledge.

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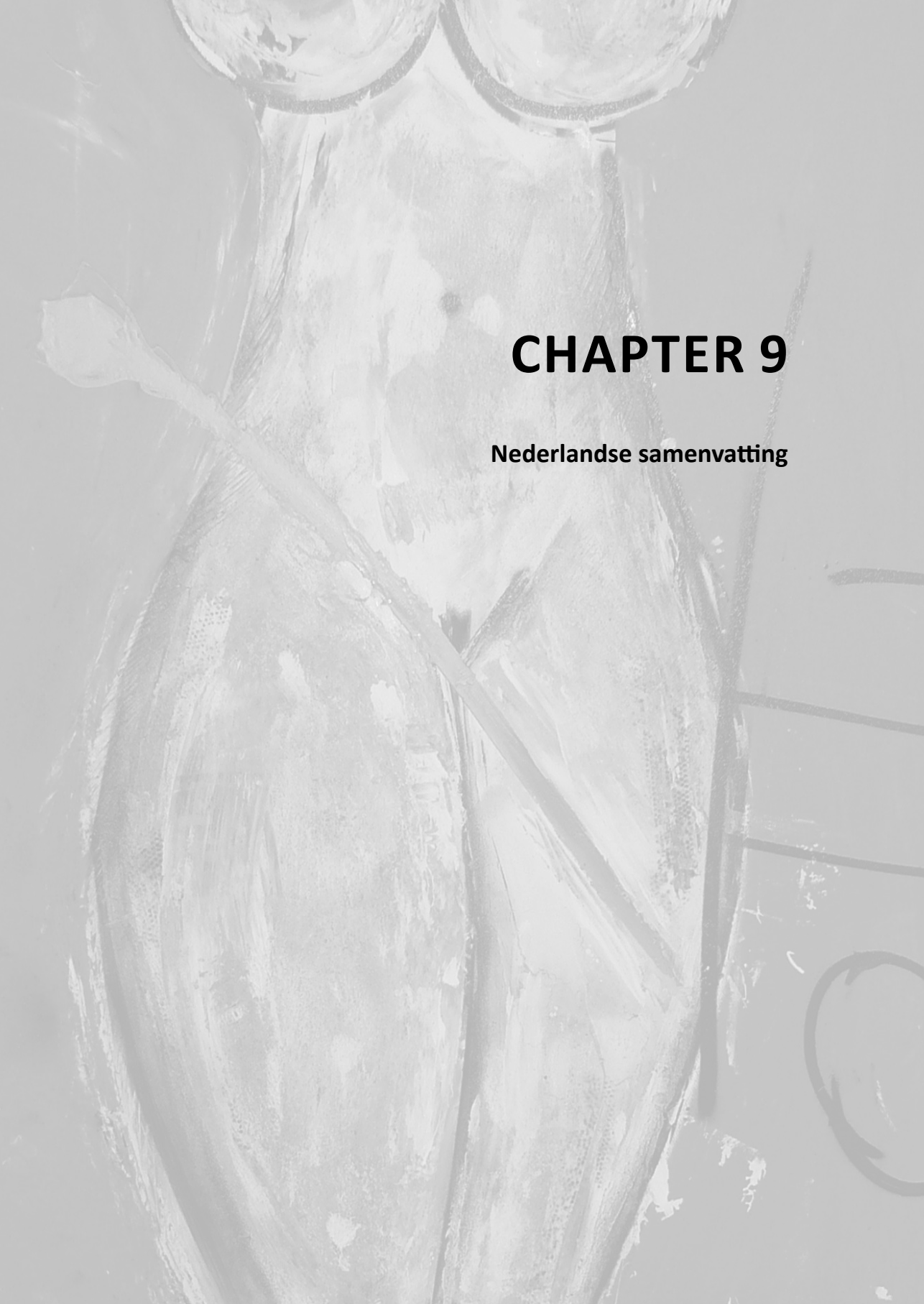


CHAPTER 9

Nederlandse samenvatting

In **Hoofdstuk 1** wordt een algemene introductie gegeven over het voorkomen van baarmoederhalskanker in Nederland en wereldwijd. Vooral in Afrika, Azië en Zuid Amerika worden hoge incidenties gevonden. Uit epidemiologisch onderzoek en klinische data blijkt dat de ontwikkeling van baarmoederhalskanker een multifactorieel proces is waarin "high-risk" humaan papilloma virus (HPV) een centrale rol speelt. Waarschijnlijk wordt 75-80% van alle seksueel actieve mensen ooit met HPV besmet. Geschat wordt dat 90% van deze personen het HPV weet te elimineren, waarbij factoren als roken en het gebruik van immuunsuppressiva van groot belang lijken te zijn. Zodra het HPV zich heeft ingebouwd in het menselijk DNA treedt er expressie op van vroege HPV oncogenen E5, E6 en E7, welke verantwoordelijk zijn voor de ontwikkeling van pre-maligne voorstadia en uiteindelijk baarmoederhalskanker. Zodra baarmoederhalskanker is vastgesteld, is het afhankelijk van het FIGO stadium en het wel of niet aanwezig zijn van vergrote lymfklieren, waaruit de behandeling kan bestaan. In het algemeen worden lagere stadia behandeld met chirurgie en de hogere stadia met radiotherapie, eventueel in combinatie met chemotherapie of hyperthermie. Baarmoederhalskanker cellen zijn, evenals stromacellen en cellen behorend tot het immuunsysteem, in staat tot de aanmaak en verspreiding van cytokines. Dit zijn kleine signaalmoleculen welke in lage concentraties geproduceerd worden. Zij worden onderverdeeld in subcategorieën analoog aan de positie op het humaan DNA en het effect is vaak lokaal, maar kan ook op afstand zijn. Specifieke cytokines, onderzocht in dit proefschrift, worden beschreven waarbij vooral aandacht wordt besteed aan de ontsteking bevorderende cytokines. Baarmoederhalskanker cellen worden vaak omgeven door een ontstekingsinfiltraat, bestaande uit voornamelijk lymfocyten, macrofagen, dendritische cellen (DC) en in mindere mate natural killer (NK) cellen. De interactie tussen het ontstekingsinfiltraat, het tumorstroma bestaande uit onder meer fibroblasten en bloed- en lymfevaten, en de tumorcellen (het tumor micromilieu) bepaalt of dit leidt tot antitumor activiteit of juist tot tumor progressie.

Chemokines zijn een specifiek soort cytokine die geproduceerd worden door verschillende celtypen, waardoor in het bijzonder ontstekingscellen zoals monocyten, welke in het weefsel differentiëren tot macrofagen, worden

aangetrokken. In **Hoofdstuk 2** wordt de relatie tussen de mate van CCL2, ook wel monocyte chemotactic protein (MCP)-1 genoemd, expressie in de tumorcellen en de aanwezigheid van tumor-geassocieerde macrofagen (TAM) onderzocht. Er is, mede door gebrek aan een goedwerkend antilichaam op paraffine coupes, gekozen voor het ontwikkelen van een mRNA-probe dat niet de hoeveelheid CCL2 als eiwit, maar de hoeveelheid *CCL2* als mRNA aangeeft met behulp van een mRNA-*in situ* hybridisatie (RISH) methode. De gebruikte scoringsmethode, waarbij de intensiteit van de aankleuring en het percentage positieve tumorcellen wordt opgeteld, werd gevalideerd met behulp van een real-time quantitative polymerase chain reaction (qRT-PCR). De expressie niveaus (geen, lage en hoge expressie) berekend met deze scoringsmethode correleerden goed met de uitslagen van de qRT-PCR. Toename van expressie van *CCL2* correleerde zeer sterk met een toename van het aantal TAM. Ook klinisch/pathologische parameters werden vergeleken met het expressie niveau van *CCL2* waarbij er vooral een correlatie aanwezig was tussen *CCL2* expressie en (ziektevrije) overleving. Patiënten die geen *CCL2* tot expressie brachten bleken een betere overleving te hebben. Door middel van “loss of heterozygosity” (LOH) onderzoek van het gebied waarin het gen, verantwoordelijk voor de expressie van CCL2, is gelegen (chromosoom 17), werd duidelijk dat bij 5 van de 6 patiënten waar *CCL2* in de coupes niet aantoonbaar was, er sprake was van verlies van (een deel) van dit gebied van chromosoom 17.

Behalve CCL2 zijn ook andere cytokines van invloed op de samenstelling van het ontstekingsinfiltraat dat vaak in het tumorstroma van baarmoederhalskanker aanwezig is. In **Hoofdstuk 3** werd daarom de rol van een aantal relevante ontsteking bevorderende cytokines in baarmoederhalskanker onderzocht, te weten granulocyte-monocyte colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α en interleukine (IL)-12 waarbij ook in deze studie gebruik werd gemaakt van de RISH. Deze cytokines spelen een belangrijke rol in het activeren, differentiëren en migreren van antigeen-presenterende cellen (APC). Ook deze cytokines werden tot expressie gebracht door baarmoederhalskanker cellen en de mRNA expressie niveaus werden vergeleken met aan- dan wel afwezigheid en/of aantal APC in de tumor. APC is een verzamelnaam voor een grote groep verschillende cellen betrokken bij het

afweersysteem, waarbij fragmenten van micro-organismen of antigenen worden opgenomen en ter plaatse of in een lymfklier gepresenteerd worden aan helper T-cellen voor een effectieve immuunrespons. De APC fungeren als boodschappers tussen de natuurlijke en verworven immuniteit. In deze studie werd gekeken naar TAM, cellen van Langerhans (LHC) en volwassen dendritische cellen (DC). TAM kwamen zowel intra-epitheliaal als in het tumorstroma voor terwijl LHC in het bijzonder intra-epitheliaal en DC nagenoeg uitsluitend in het tumorstroma werden aangetoond. Er bleek een positieve correlatie te bestaan tussen de hoeveelheid TAM en de hoeveelheid DC. Beide soorten APC waren ook positief gecorreleerd met *GM-CSF* en *TNF-α. IL-12* was positief gecorreleerd met het aantal TAM en *TNF-α* was juist negatief gecorreleerd met het aantal LHC. Een hoge expressie of juist afwezigheid van de functionele subunit van *IL-12* (*IL-12p40*) bleek geassocieerd met een betere overleving. Deze studie suggereert een rol voor zowel *GM-CSF* als *TNF-α* in het rekruteren en mogelijk ook differentiëren van monocyten naar macrofagen of volwassen DC of zelfs LHC. De negatieve correlatie tussen *TNF-α* en LHC wijst juist op een rol van de *TNF-α* in de migratie van de LHC naar de regionale lymfklieren voor antigeen presentatie.

In hoofdstuk 3 is aangetoond dat afwezigheid of juist hoge IL-12p40 mRNA expressie geassocieerd is met een verbeterde overleving in baarmoederhalskanker. In **Hoofstuk 4** werd de rol van IL-12p40 (als subcomponent van IL-12 en IL-23) en IL-6 nader onderzocht. IL-12 is in staat om leukocyten als $CD8^+$ T lymfocyten en NK cellen te activeren wat kan leiden tot een antitumor respons. IL-23 speelt een belangrijke rol in de IL-17/IL-23 route, resulterend in de aanmaak van T helper (T_H)17 lymfocyten welke IL-17 kunnen produceren. De rol van IL-17 bij het ontstaan en de groei van kanker lijkt een tweeslachtige: IL-17 is in staat tot zowel tumorprogressie als tumorregressie, waarbij het netto effect mogelijk afhankelijk is van meerdere factoren. Uit onze data kan worden afgeleid dat, bij een lage hoeveelheid *IL-12p40* het biologische effect van IL-23 dominant is, terwijl bij een hoge hoeveelheid *IL-12p40* het effect van IL-12 juist de overhand heeft. Dit resulteert in respectievelijk een minder goede en een betere overleving. Naast IL-23 lijken ook IL-1 en IL-6 een belangrijke rol te spelen bij het stimuleren van de IL-17/IL-23 route. In onze studie is er geen relatie tussen IL-1 en

ziektevrije overleving. IL-6 producerende cellen werden voornamelijk aangetoond in het tumorstroma. Indien de groep baarmoederhalskanker patiënten werd verdeeld in patiënten met een hoog aantal IL-6 producerende cellen en patiënten met een laag aantal IL-6 producerende cellen, bleken patiënten met een laag aantal IL-6 producerende cellen een betere prognose te hebben vergeleken met de patiënten met een hoog aantal IL-6 producerende cellen. Indien de groep patiënten met een hoog aantal IL-6 producerende cellen in het tumorstroma verder werd onderverdeeld in een groep met expressie van *IL-12* en een groep met expressie van *IL-23*, dan liet de combinatie van hoog aantal IL-6 producerende cellen in het tumorstroma in combinatie met een lage expressie van *IL-12p40* een duidelijk slechtere ziektevrije overleving zien. De IL-17/IL-23 route lijkt, naar aanleiding van deze resultaten, een immunosuppressieve rol te vervullen binnen stadium IB-IIA baarmoederhalskanker.

De samenstelling van het tumorstroma is van grote invloed op het biologisch gedrag van kanker. Behalve ontstekingsinfiltraat is ook een balans nodig tussen aanvoer van zuurstof en voedingsstoffen en afvoer van afvalstoffen. Tumorgroei en het ontwikkelen van uitzaaiingen (metastasering) heeft vaatnieuwvorming (angiogenese) nodig om deze balans veilig te stellen. In **Hoofdstuk 5** wordt de rol van angiogenese onderzocht in baarmoederhalskanker. De prikkel voor angiogenese kan liggen in zuurstoftekort (hypoxie) in de centra van (grotere) tumorvelden, maar zou ook al in een vroeg stadium door de tumor opgestart kunnen worden door productie van pro-angiogenetische factoren. Als een tumor een grootte van 1 mm^3 overschrijdt is diffusie van voedingsstoffen en afvalstoffen onvoldoende en is vaatnieuwvorming nodig voor verdere groei. Angiogenese is een proces waarbij veel factoren een rol spelen. In deze studie werden immuunhistochemische kleuringen verricht om het aantal nieuwgevormde bloedvaten (CD105 positief) ten opzichte van het totaal aantal aanwezige bloedvaten (CD31 positief) te bepalen, waarbij niet alleen werd gelet op het aantal bloedvaten, maar ook de positie van deze vaten in de tumor. Eén van de belangrijkste factoren voor vaatnieuwvorming is vascular endothelial growth factor-A (VEGF-A) waarvoor zowel een RISH als een immuunhistochemische eiwitkleuring werd verricht. Daarnaast werden andere factoren betrokken bij de angiogenese onderzocht, zoals basic fibroblast growth factor (bFGF), plasminogen

activator inhibitor-1 (PAI-1), matrix metalloproteinase-2 (MMP-2) en transforming growth factor (TGF)-*β*1. Het aantal CD31- en CD105 positieve bloedvaten bleek onderling positief gecorreleerd: hoe groter het aantal bloedvaten in de tumor, hoe meer CD105 positieve bloedvaten aanwezig waren. Zowel bFGF- als VEGF-A-producerende cellen werden voornamelijk gevonden in het tumorstroma en indien tumoren VEGF-A produceerden was dit vooral aanwezig op de grens tussen tumorepitheel en tumorstroma, de plaats waar ook de bFGF producerende cellen voornamelijk te vinden waren. Er was geen correlatie tussen het aantal CD105 positieve bloedvaten en aantal bFGF- of VEGF-A- producerende cellen. Er was wel een correlatie tussen het aantal CD105 positieve bloedvaten en de *VEGF-A* expressie door tumorcellen. Verrassend bleek dat de productie van VEGF-A eiwit niet overeen kwam met *VEGF-A* mRNA in onze kleuringen. TAM staan bekend om de productie van angiogenese bevorderende signaalmoleculen. Er bleek een positieve correlatie tussen het aantal TAM en het aantal CD105 positieve bloedvaten te zijn waarbij opgemerkt dient te worden dat dit het totaal aantal TAM in de tumor betreft, dus zowel degene tussen de epitheliale tumorcellen als degene in het tumorstroma. Het aantal TAM correleerde tevens met de intensiteit van aankleuring van het integrine *αvβ*6, een receptor betrokken bij proliferatie en migratie van tumorcellen en activatie van TGF-*β*. Zowel CD31- als CD105 positieve vaten bleken een positieve relatie te hebben met lymfkliermetastasen, waarbij CD105 positieve vaten in het tumorstroma ook positief gecorreleerd waren met het aantal metastasen bevattende lymfklieren. De aanwezigheid van het aantal CD105 positieve vaten ín de epitheliale tumorvelden bleek negatief gecorreleerd met ziektevrije overleving. In deze studie werd geconcludeerd dat CD105 gebruikt kan worden als een (slechte) prognostische factor waarbij de positie van deze vaten in de tumor van belang is.

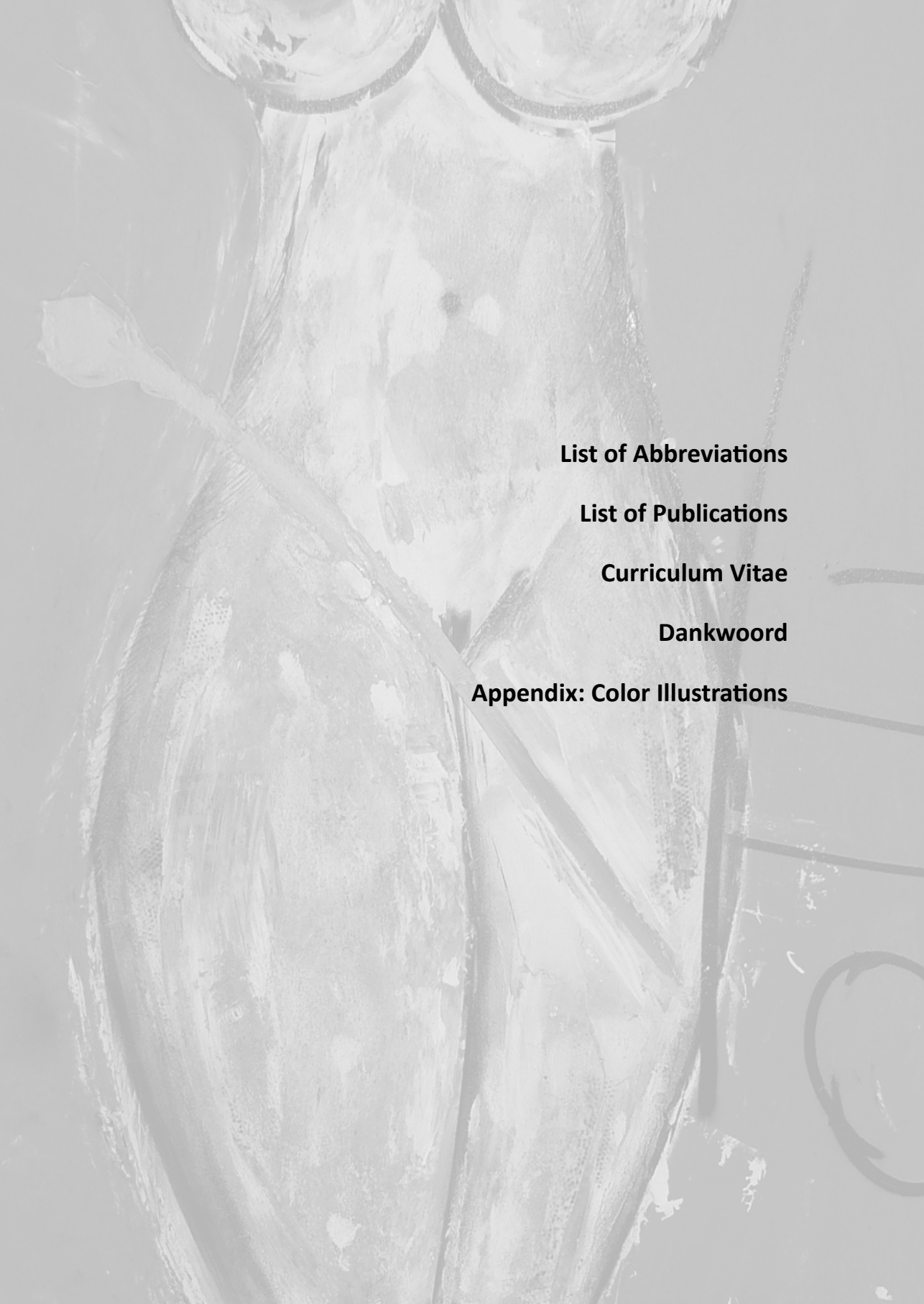
De grens tussen tumorepitheel en tumorstroma lijkt van groot belang in de expansie van baarmoederhalskanker. Dit is het gebied waar VEGF-A werd aangetroffen en bFGF producerende cellen voornamelijk werden aangetoond. Het is ook het gebied waar ruimte gemaakt dient te worden voor groei en angiogenese waarvoor herstructurering van het tumorstroma nodig is. **Hoofdstuk 6** handelt over de expressie van matrix metalloproteinase (MMP)-7 in baarmoederhalskanker. MMP zijn eiwit afbrekende

enzymen, betrokken bij afbraak en herstructurering van extracellulaire matrix en basaal membranen. MMP kunnen daardoor invloed hebben op tumorprogressie door ruimte te creëren voor expansie van tumorcellen en vaatnieuwvorming, leidend tot groei en mogelijkheid tot metastasering. Zuurstoftekort in tumorweefsel en aanwezigheid van VEGF zijn signalen die kunnen leiden tot productie van MMP-7, een MMP dat de mate waarin cellen aan elkaar gehecht zijn (cel-cel contact) beïnvloed en effect heeft op het activeren van andere MMP en het vrijmaken/ splitsen van andere bioactieve moleculen. Productie van MMP-7 werd aangetoond in de bekledende cellen van bloedvaten (endotheel) in verschillende soorten kanker waaronder baarmoederhalskanker. Ook werd aangetoond dat het voorkomen van MMP-7 in de endotheelcellen overeenkomt met vaatnieuwvorming door middel van een CD105 kleuring. *In vitro* onderzoek bevestigde dit: remming van MMP-7 leidde tot remming van angiogenese en expressie van MMP-7 werd bevorderd door de toevoeging van VEGF. MMP-7 bleek in deze studie een slechte prognostische factor: hoge aanwezigheid van MMP-7 in endotheelcellen correleerde met een slechtere overleving.

Behalve MMP-7 is ook versican in staat tot het destabiliseren van cel-cel contacten. Hierdoor is ook versican in staat invloed uit te oefenen op tumorprogressie en angiogenese. In **Hoofdstuk 7** werd de aanwezigheid van versican en de relatie tussen aanwezigheid van versican en de aanwezigheid van verschillende typen ontstekingscellen onderzocht. Versican is een onderdeel van de extracellulaire matrix en behoort tot de familie van de proteoglycanen. Naast cel-cel contact en migratie van cellen speelt versican ook een rol bij de ontstekingsreactie door interactie met chemokines die een rol spelen bij het aantrekken van ontstekingscellen. In deze studie werd de aanwezigheid, type en locatie van versican in baarmoederhalskanker bepaald, als ook de relatie met de aanwezigheid van verschillende ontstekingsceltypen. Bij baarmoederhalskanker blijkt versican voornamelijk in het tumorstroma aanwezig te zijn, met name op de grens van tumorcellen en tumorstroma, en rondom bloedvaten. Versican bleek vooral geproduceerd te worden door myofibroblasten (spiercellen) in het tumorstroma. Van de verschillende vormen waarin versican voor kan komen was V0 het meest prominent aanwezig. Een hoge expressie van versican bleek

gecorreleerd te zijn met een lager aantal intra-epitheliale $CD8^+$ T lymfocyten. Dit effect is mogelijk het gevolg van het binden (en daarmee inactiveren) van cytokines die normaal T lymfocyten aantrekken. Verder heeft versican een positieve relatie met een grotere invasiediepte (≥15 mm) en een hogere kans op de aanwezigheid van tumorcellen in de parametria, mogelijk door enerzijds het bevorderen van de mobiliteit van baarmoederhalskanker cellen en anderzijds verlaging van de binding van deze cellen aan andere tumorcellen en omgevende tumorstroma cellen. Er werd geen relatie tussen expressie van versican en overleving gevonden.

In **Hoofdstuk 8** wordt nader ingegaan op de mogelijkheden van diagnostiek naar (pre)maligne afwijkingen van de baarmoederhals (het afnemen van uitstrijkjes, self-sampling) en de toekomst van behandeling of voorkomen van baarmoederhals afwijkingen, waarin vaccinatie een grote rol zal spelen en uiteindelijk een afname van zowel baarmoederhalskanker als ook vulva-, vagina- en anuskanker zou kunnen bewerkstelligen. Momenteel zijn er 2 preventieve HPV vaccins op de markt, te weten Gardasil® en Cervarix®. Vaccinatie wordt in Nederland sinds 2009 aangeboden aan meisjes en jonge vrouwen van 12 t/m 16 jaar waarbij primaire preventie wordt nagestreefd. Het in Nederland momenteel gebruikte vaccin Cervarix® is werkzaam tegen type HPV 16 en 18. Nadat een HPV-infectie heeft plaatsgevonden is primaire preventie (het voorkómen van een infectie) niet meer mogelijk. Om het virus effectief te bestrijden wordt momenteel veel onderzoek gedaan naar het ontwikkelen van een therapeutisch vaccin. Deze onderzoeken richten zich met name op versterking van de T-cel reactie tegen specifieke eiwitten welke in een geïnfecteerde cel aanwezig zijn. Ook zijn er momenteel diverse studies waarin het immuun modulerende effect van (onder andere) anti-VEGF en anti-IL6 wordt onderzocht. Wellicht dat de incidentie van, door HPV-geïnduceerde, kanker en voorstadia hiervan verder af zal nemen door preventieve vaccinatie. In geval van baarmoederhalskanker kan in de toekomst door analyse van een biopt van de tumor mogelijk een specifiek op die tumor gerichte behandeling (personalized medicine) worden voorgesteld.



ABBREVIATIONS

ANOVA	analysis of variance
APAAP	alkaline phosphatase anti-alkaline phosphatase
APC	antigen presenting cells
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CAF	cancer-associated stromal fibroblasts
Caspase	cysteine-dependent aspartate-directed proteases
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) recepor
CD	cluster of differentiation
CIN	cervical intraepithelial neoplasia
CRI	cancer-related inflammation
CSF-1	colony stimulating factor-1
CSPG	chondroitin sulphate proteoglycan
CTGF	connective tissue growth factor
CXC	chemokine CXC motif
DC	dendritic cells
DCreg	regulatory DC
DFS	disease-free survival
DIG	digoxigenin
E	early
E6AP	E6 associated protein
ECM	extracellular matrix
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour(s)
HPF	high-power field of view
HPV	human papilloma virus
HRP	horseradish peroxidase

HSIL	high-grade squamous intraepithelial lesion
IFN	interferon
IL	interleukin
IL-6R	IL-6 receptor
IL-23R	IL-23 receptor
JAK	Janus kinase
L	late
lDC	lymphoid DC
LHC	Langerhans' cells
LLETZ	large loop excision of the transformation zone
LOH	loss of heterozygosity
LPS	lipopolysaccharide
LSIL	low-grade squamous intraepithelial lesion
M-CSF	macrophage colony-stimulating factor
MAb	monoclonal antibody
MAPK	mitogen-associated protein kinase
MCP-1	monocyte chemotactic protein-1
mDC	myeloid DC
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NK	natural killer cells
on	overnight
OS	overall survival
PAI-1	plasminogen activator inhibitor-1
Pap	Papanicolaou
PBA	phosphate-buffered saline containing 1% bovine serum albumin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGE	prostaglandin E

PI	propidium iodide
PI3K	phosphatidyl inositol-3'-kinase
pRb	retinoblastoma protein
RISH	RNA-in situ hybridization
RORγt	related orphan receptor γt
RT-PCR	real-time quantitative polymerase chain reaction
qRT-PCR	quantitative RT-PCR
SD	standard deviation
SLP	synthetic long peptide
SSC	saline-sodium citrate
STAT	signal transducer and activator of transcription\
STS	sequence tagged site
TAM	tumor-associated macrophages
TGF-β	transforming growth factor-β
T_H	T helper lymphocyte
TIMP-2	tissue inhibitor of metalloproteinase-2
TNF-α	tumor necrosis factor-α
TNFR	tumor necrosis factor receptor
TNM	cancer staging system: tumor size, regional lymph node, distant metastasis
TRAF	TNFR-associated factor
TRAIL-R1	TNF-related apoptosis-inducing ligand-receptor 1
Treg	regulatory T cells
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VIA	visual inspection with acetic acid
WHO	World Health Organization

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CURRICULUM VITAE

Henry Zijlmans, auteur van dit proefschrift, werd op 18 november 1963 geboren in Eindhoven. In 1982 behaalde hij zijn HAVO diploma aan het Eindhovens Protestants Lyceum te Eindhoven. Dat jaar startte hij met middelbaar laboratorium onderwijs, gevolgd door hoger laboratorium onderwijs aan de Hogeschool West-Brabant te Etten-Leur, afstudeerrichting histologie. De stage en afstudeeropdracht werden verricht bij de afdeling Celpathologie van het Academisch Medisch Centrum te Amsterdam, waar onder leiding van dr. F.M. van den Berg onderzoek werd verricht naar detectie van *C. pylori* in paraffine coupes met behulp van DNA-*in situ* hybridisatie. Na het vervullen van de militaire dienstplicht werkte hij van 1990 tot 1999 als research analist bij de afdeling Cytochemie & Cytometrie van het Sylvius Laboratorium te Leiden, waar hij onderzoek deed naar het gebruik van kleine anorganische fosfordeeltjes als immunocytochemische marker, onder leiding van prof. dr. H.J. Tanke. In 1991 koos hij alsnog voor de studie geneeskunde waar hij datzelfde jaar werd ingeloot. Dit werd omgezet in een schaduwplaats aangezien nog natuurkunde en scheikunde op VWO-niveau gehaald dienden te worden. In 1992 werd met de studie geneeskunde gestart aan de Rijksuniversiteit Leiden. In 1995 verrichtte hij een klinische stage bij de afdeling Traumatología van Hospital Obrero te La Paz, Bolivia. De co-schappen werden afgesloten met een keuze co-schap Anaesthesiologie in het Leids Universitair Medisch Centrum.

In 1999 behaalde hij zijn artsenbul en startte hij als AGNIO (assistent geneeskundige niet in opleiding) op de afdeling Gynaecologie en Verloskunde van het Eemland Ziekenhuis, locatie St. Elisabeth te Amersfoort, gevolgd door een AGNIO positie op de afdeling Gynaecologie en Verloskunde van het Westeinde Ziekenhuis te Den Haag. In 2001 begon hij als AGIKO (assistent geneeskundige in opleiding tot klinisch onderzoeker) met promotie onderzoek bij de afdelingen Gynaecologie en Pathologie van het Leids Universitair Medisch Centrum, uitgevoerd onder leiding van prof. dr. G.J. Fleuren, prof. dr. G.G. Kenter en dr. A. Gorter. In 2004 begon hij met de opleiding tot gynaecoloog in het Leids Universitair Medisch Centrum (opleiders prof. dr. H.H.H. Kanhai en prof. dr. G.G. Kenter) en het Medisch Centrum Haaglanden te Den Haag (opleider dr. P.J. Dörr) wat in augustus 2009 werd afgerond. Van september 2009 tot

en met november 2011 was hij fellow Gynaecologische Oncologie in het Universitair Medisch Centrum Groningen, onder leiding van prof. dr. A.G.J. van der Zee. Sinds december 2011 is hij werkzaam als gynaecologisch oncoloog in het Centrum voor Gynaecologische Oncologie Amsterdam (CGOA), locatie Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis (NKI-AvL).

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Een proefschrift is het resultaat van samenwerking. Ik wil graag de mensen bedanken die hebben bijgedragen aan de totstandkoming van dit proefschrift. Allereerst alle coauteurs, betrokken bij de hoofdstukken. Dank voor jullie discussies, kritische kanttekeningen, opbeurende kritiek en vooral ook het vertrouwen.

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Stafleden, A(N)IOS, verpleegkundigen en (poli)assistenten van de verschillende ziekenhuizen waar ik in deze periode werkzaam ben geweest: LUMC (met name de wekelijkse AGIKO lunches waren altijd nuttig maar vooral ook een humorvol klankbord), Medisch Centrum Haaglanden (met name Joep en Marjolein, dank voor jullie betrokkenheid en grote steun voor deze coassistent/ANIOS/AIOS/gynaecoloog) en het UMCG (Ate, Hans, Henriëtte en Marjan: dank voor een geweldige tijd in Groningen en de mogelijkheid om de wetenschappelijke draad weer op te pakken). Ook kamergenoten uit het UMCG (Ayten, Maaike, Marinka, Maureen, Moira en Nathalie): geweldig om een kamer met mede fellows te delen.

Hans Tanke, jij hebt eigenlijk aan de basis gestaan van mijn opleiding tot gynaecologisch oncoloog, al wisten we dat toen nog niet. Door mijn baan als research analist naar 50% te verlagen en werkbesprekingen af te stemmen op mijn diensten schema als coassistent was het voor mij mogelijk om de studie Geneeskunde te volgen. En ook al noemde je het een ‘win-win’ situatie, heel veel dank hiervoor. De collega’s van Vakgroep Cytochemie & Cytometrie van het Sylvius Laboratorium, in het bijzonder Annemarie, Berna, Frans, Irma, Joop, Karien, Richard en Rob: bedankt voor een fantastische leerschool gecombineerd met veel plezier.

Ook familie en vrienden bedankt voor steun door de jaren heen. Door mijn chronische drukte is er nogal eens geschoven met afspraken, bezoekjes en bijeenkomsten. Dank voor jullie begrip.

Beste Merryn en Onno, dat jullie bereid zijn om mijn paranimfen te zijn is voor mij ongelofelijk waardevol. Onno: één terrasje in Eindhoven in 1990 heeft geleid tot de studie Geneeskunde, specialisatie tot gynaecologisch oncoloog en dit proefschrift. Het lijkt me een goed idee als je nu ook bij de afronding bent. Merryn, door afstand en tijd zien en spreken we elkaar minder dan ik zou willen. Toch is iedere keer dat we elkaar treffen als vanouds.
En tenslotte Jaklien: we kennen elkaar nu meer dan 20 jaar waarbij ieder jaar drukker lijkt te worden. Zeker de laatste jaren waren erg druk met schrijven, diensten en opleiding. Je hebt mij vaak de ruimte gegeven om dit alles te kunnen doen naast je eigen drukke baan en ons gezin. Dank voor alles. We gaan wederom een avontuur tegemoet. Samen met Marieke, Bas en Koen gaat dat helemaal lukken.

APPENDIX: COLOR ILLUSTRATIONS

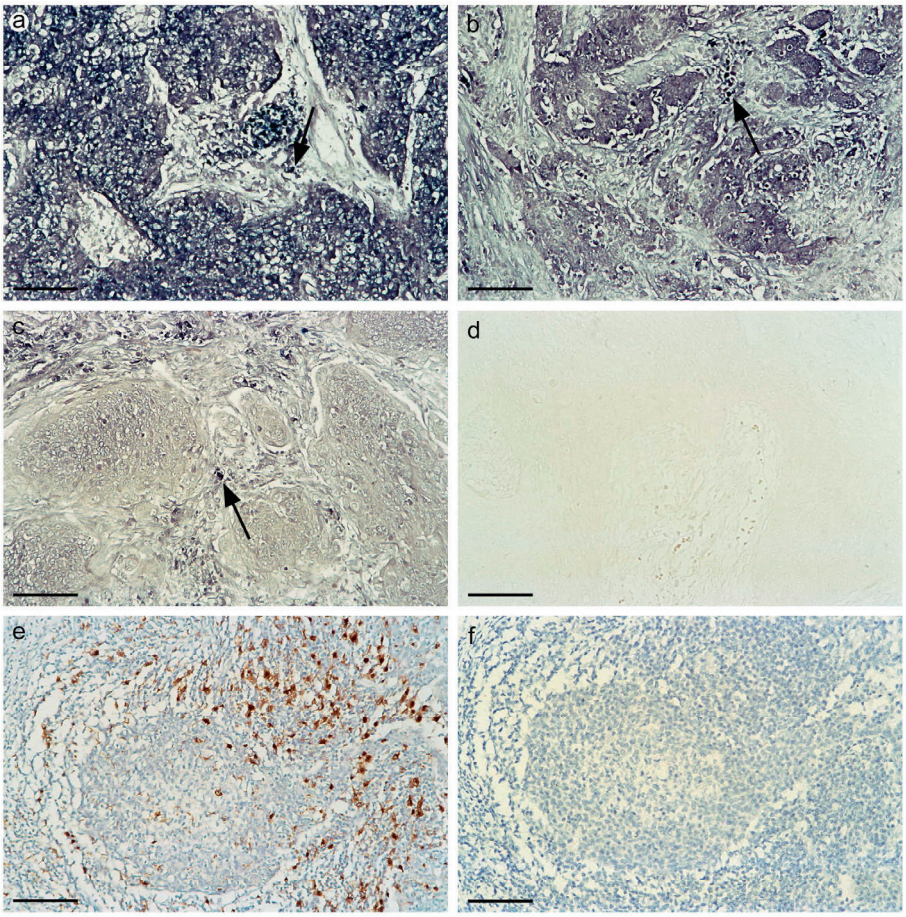


Figure 1. *CCL2* (RISH) and CD68 (immunohistochemical) staining for monocytes/macrophages in cervical carcinoma. Panels (**a**), (**b**) and (**c**) represent strong, weak and no expression of *CCL2* mRNA respectively. (**d**) Sense probe for *CCL2* (negative control). Monocytes/macrophages are indicated by arrows. Panels (**e**) and (**f**) represent positive and negative immunohistochemical staining of monocytes/macrophages (anti-CD68), respectively; sections are counterstained with Mayer's haematoxylin. (**a**-**f**) Scale bar = 100 μm.

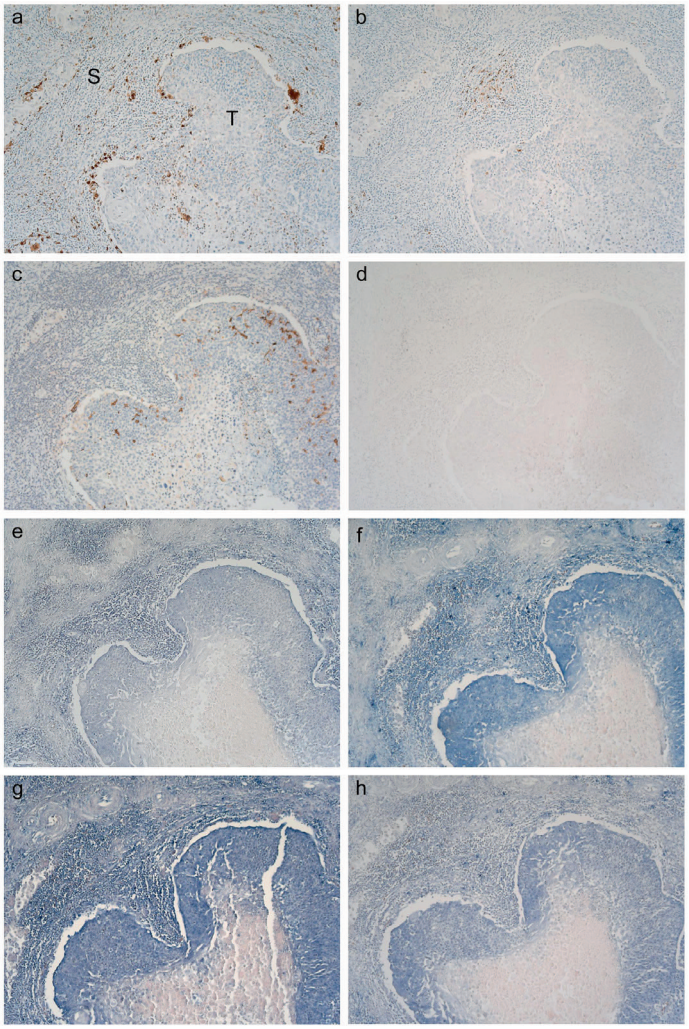


Figure 1. Immunohistochemical and RISH staining of paraffin sections of cervical carcinoma are shown. Carcinoma cells are indicated by a T, whereas S represents tumor stroma. (**a**-**c**) Immunohistochemical staining of cervical carcinoma. (**a**) Staining for monocytes and macrophages (anti-CD68). Staining is present intraepithelial as well as in the tumor stroma. (**b**) Staining for mature dendritic cells [DC (anti-DC-Lamp)] seen as nests in the tumor stroma. (**c**) Staining for Langerhans' cells [LHC (anti-CD1a)]. Staining can be seen intraepithelial. Positive cells are clearly visible by a dark-brown precipitation. Sections (**a**-**c**) are counterstained with Mayer haematoxylin. (**d**-**h**) RNA-*in situ* hybridization (RISH) for cytokines in the same cervical carcinoma sample as shown in (**a**-**c**). (**d**) Negative control. (**e**) *GM-CSF* shows a weak expression in both cervical carcinoma cells and in inflammatory cells in the tumor stroma. (**f**) *TNF-α* is strongly expressed in cervical carcinoma cells as well as in some of the cells in the tumor stroma. (**g**) *IL-12p35* is strongly expressed in the cervical carcinoma cells as well as in inflammatory cells in the tumor stroma. (**h**) *IL-12p40* shows a moderate expression in the tumor cells and a strong expression in some of the cells in the tumor stroma. Positive signal in RISH is seen as a (dark) blue precipitation. Sections (**d**-**h**) are not counterstained. Original magnification, x80.

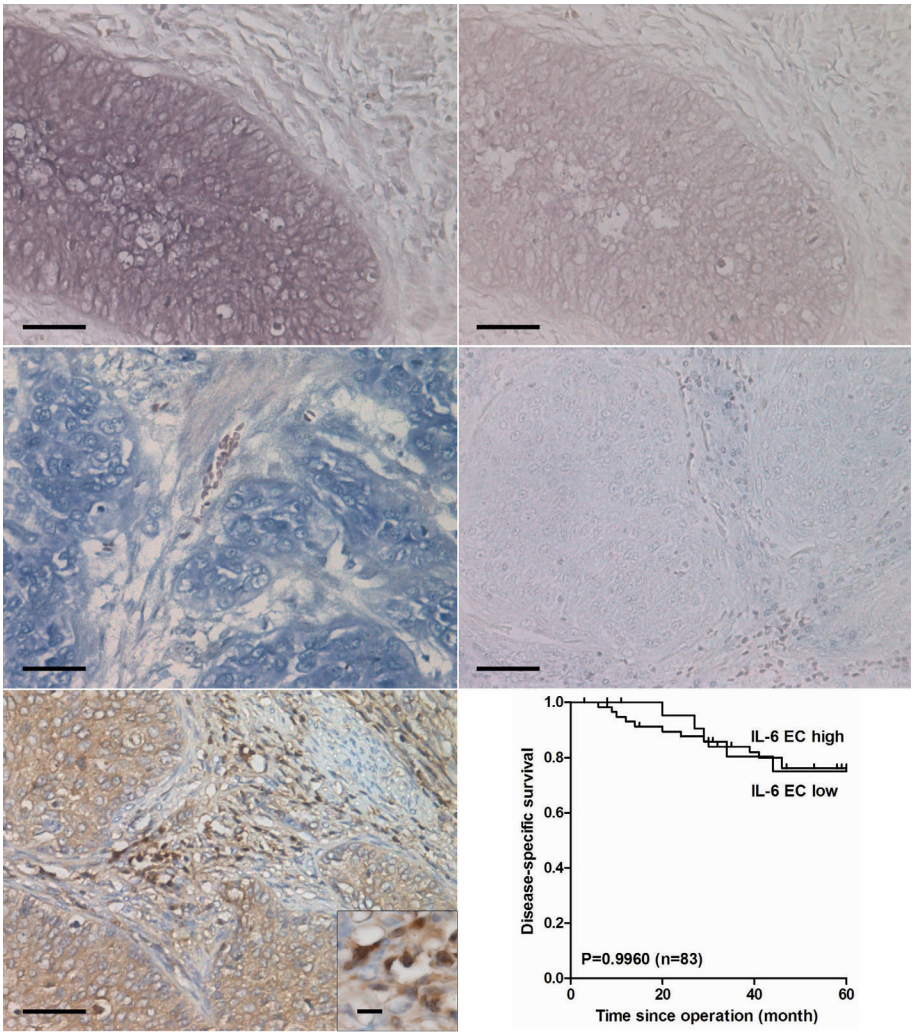
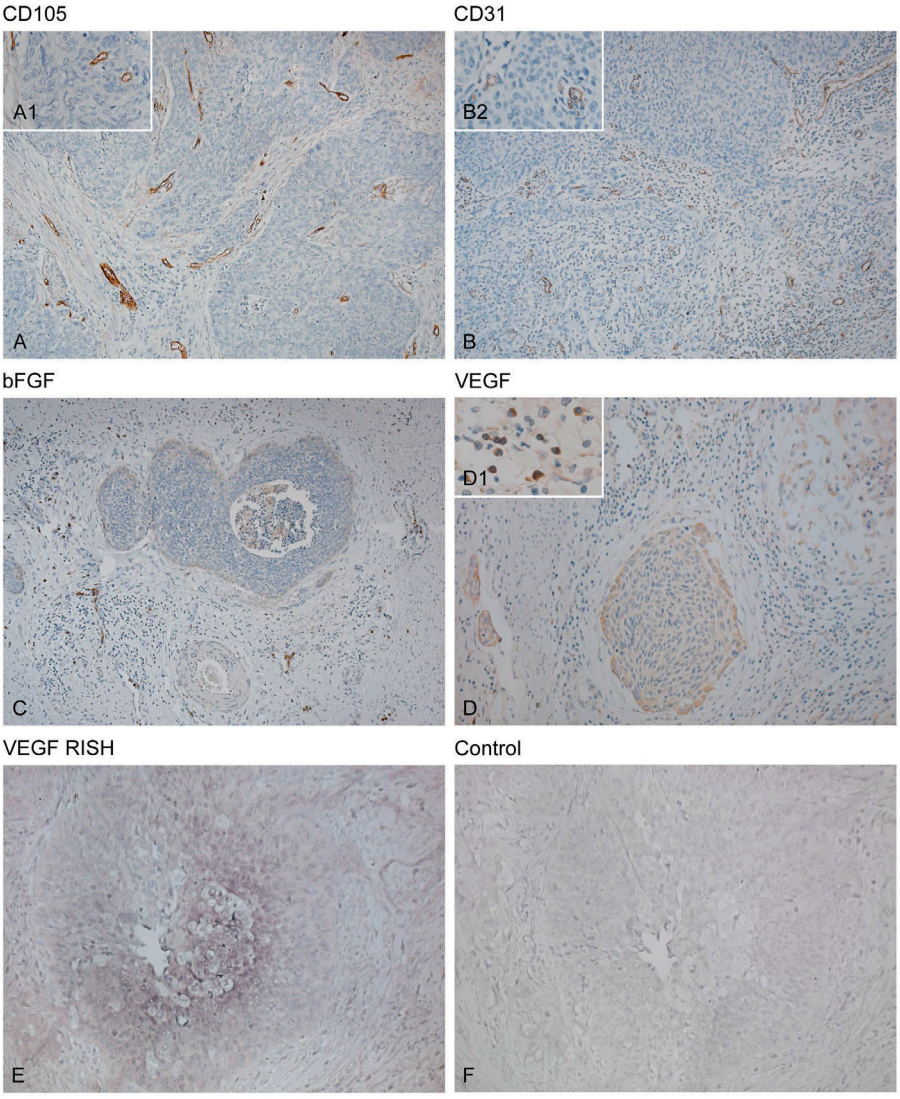


Figure 1 The expression of *IL-23p19*, *IL-12p40* and IL-6. The expression of *IL-23p19* and *IL-12p40* were determined using RNA-*in situ* hybridization (RISH) and the expression of IL-6 was determined using immunohistochemistry as described in the Materials and methods section (magnification, x250). (**A**) Cervical tumor, *IL-23p19* RISH. Tumor cells stain positive (moderate) for *IL-23p19;* (**B**) Negative (sense) control of *IL-23p19* RISH; (**C**) Cervical tumor, *IL-12p40* RISH. Tumor cells stain positive (strong) for *IL-12p40;* and (**D**) Negative (sense) control of *IL-12p40* RISH. (**E**) IL-6 staining of cervical cancer tissue. Both cells in the epithelial compartment (EC) as well as cells in the stroma express IL-6. Arrows indicate positive stromal cells. Detail (x400, magnification) of IL-6 positive cells in the stroma; and (**F**) Association between cells in the epithelial compartment with low (IL-6 EC low) and high IL-6 (IL-6 EC high) expression and disease-specific survival. No significant association between low or high IL-6 expression of the epithelial cells with disease-specific survival was observed.


CD105
A1
A
CD31
B2
B
bFGF
C
VEGF
D1
D
VEGF RISH
E
Control
F

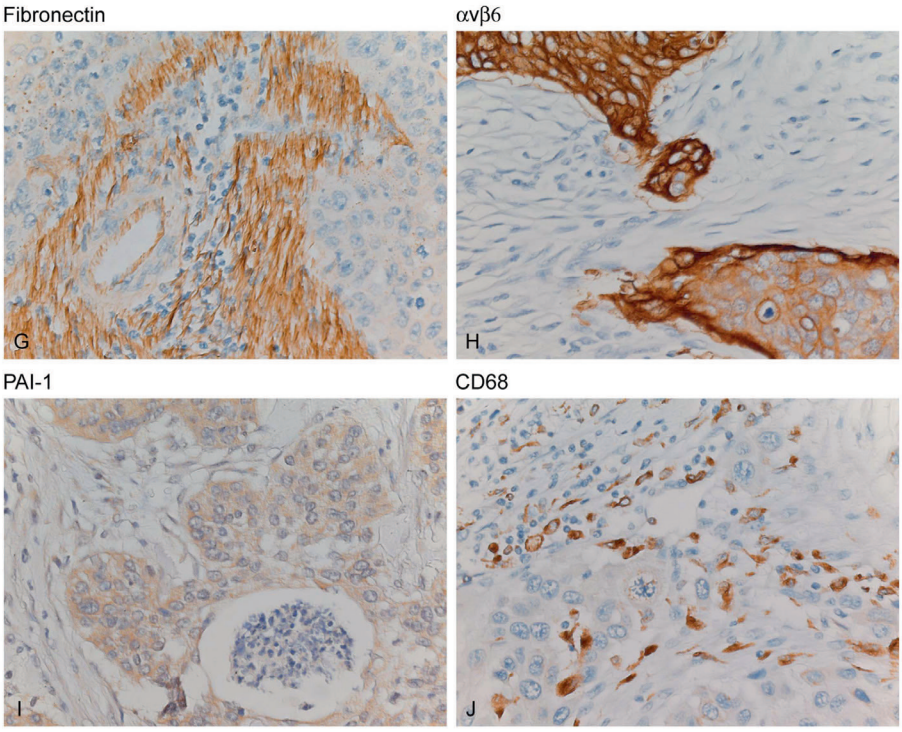


Figure 1. Expression and location of CD105- and CD31- positive vessels, bFGF, VEGF-A fibronectin, *αvβ6*, PAI-1 and CD68-positive cells.

Expression and location of CD105- and CD31- positive vessels, bFGF and VEGF-A were determined using immunohistochemistry as well as RNA-*in situ* hybridization (RISH) as described in Materials and methods section (magnification, x125). (**A**) CD105 vascular staining, (**A1**) Detail (magnification, x400) of vessels present in the tumor stroma as well as in the epithelial cell clusters. (**B**) CD31 vascular staining, (**B1**) detail (magnification, x400) of vessels present in the tumor stroma as well as in the epithelial cell clusters. (**C**) bFGF, positive staining of the border of the epithelial cell clusters and cells in the stromal compartment. (**D**) VEGF-A immunohistochemical staining with increased positive staining of the borders of the epithelial cell clusters, (**D1**) detail (magnification, x400) of VEGF-A positive stromal cells. (**E**) *VEGF-A* RISH with weak cytoplasmic staining of the epithelial cell clusters. (**F**) Negative (sense) control of *VEGF-A* RISH. Expression and location of fibronectin, *αvβ6*, PAI-1 and CD68-positive cells (magnification, x400). (**G**) fibronectin, positive staining (>75%) of the stromal compartment. (**H**) *αvβ6*, positive staining (strong intensity; of the border) of the epithelial cell clusters. (**I**) PAI-1, positive staining of the epithelial cell clusters. (**J**) Positive staining of CD68-positive cells in the stromal compartment and in the epithelial cell clusters.

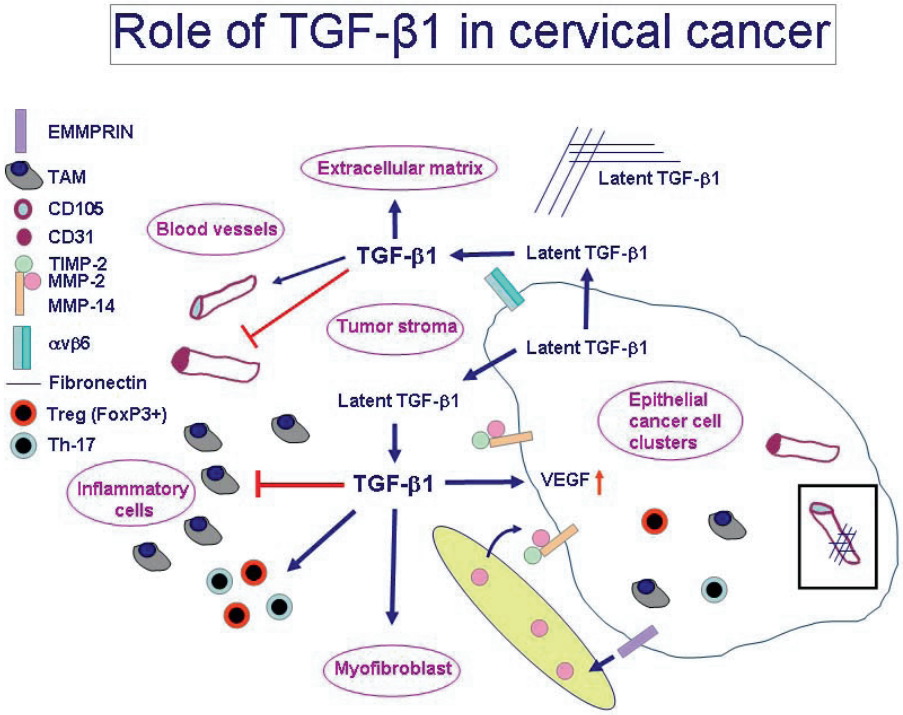


Figure 7 Role of TGF-β_1 in cervical cancer.
Cervical tumors are composed of malignant epithelial cells, tumor stroma, comprising the tumor vasculature extra cellular matrix and (myo)fibroblasts, and an inflammatory infiltrate. Latent TGF-β_1 is produced amongst others by cervical cancer cells, secreted and stored in the extracellular matrix. TGF-β_1 can be activated by the epithelial cell specific integrin $\alpha v\beta 6$ or by matrix metalloproteinases (MMP), especially active MMP-2 in complex with MMP-14 and TIMP-2 on the cell membrane of cervical cancer cells at the epithelial cell-stroma border. Active TGF-β_1 induces VEGF in the epithelial cancer cells and differentiates fibroblasts into myofibroblasts. Depending on the local concentration, active TGF-β_1, promotes endothelial proliferation and migration or promotes cytostasis and vessel maturation. Active TGF-β_1 acts as an immunosuppressor by blocking the activity of the inflammatory cells and inducing FoxP3-positive regulatory cells and T_H17 cells.

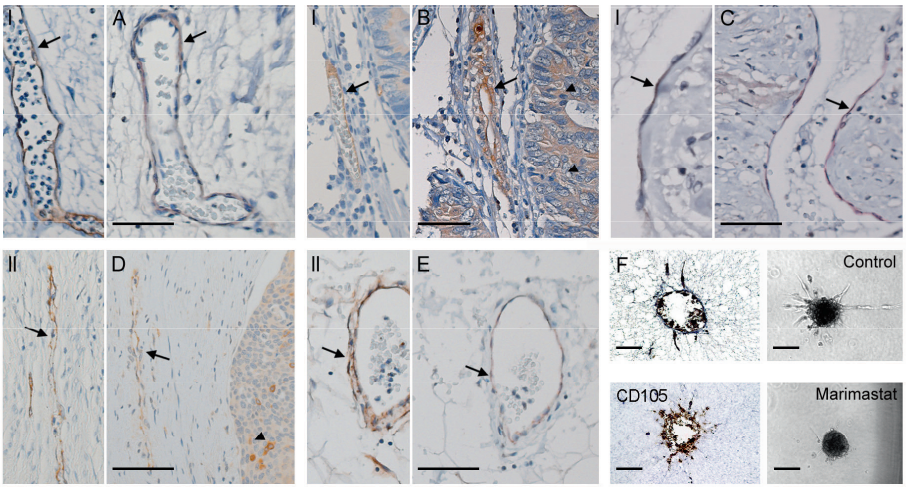


Figure 1. Immunohistochemical staining of endothelial cells for MMP-7, indicated by arrows in gastric cancer (**A**), colonic cancer (**B**), breast cancer (**C**), cervical cancer (**D**), prostate cancer (**E**), and in *in vitro* sprouting HUVEC cells (**F**). Inserts I and II indicate respectively CD34 and CD105 staining in sequential section from the same tissue. The inserts in (**F**) show VEGF-induced endothelial cell sprouting in control and Marimastat-treated HUVEC spheroids. Arrowheads indicate epithelial cell staining. Bars correspond with 100 μm in (**A**-**E**) and with 300 μm in (**F**).

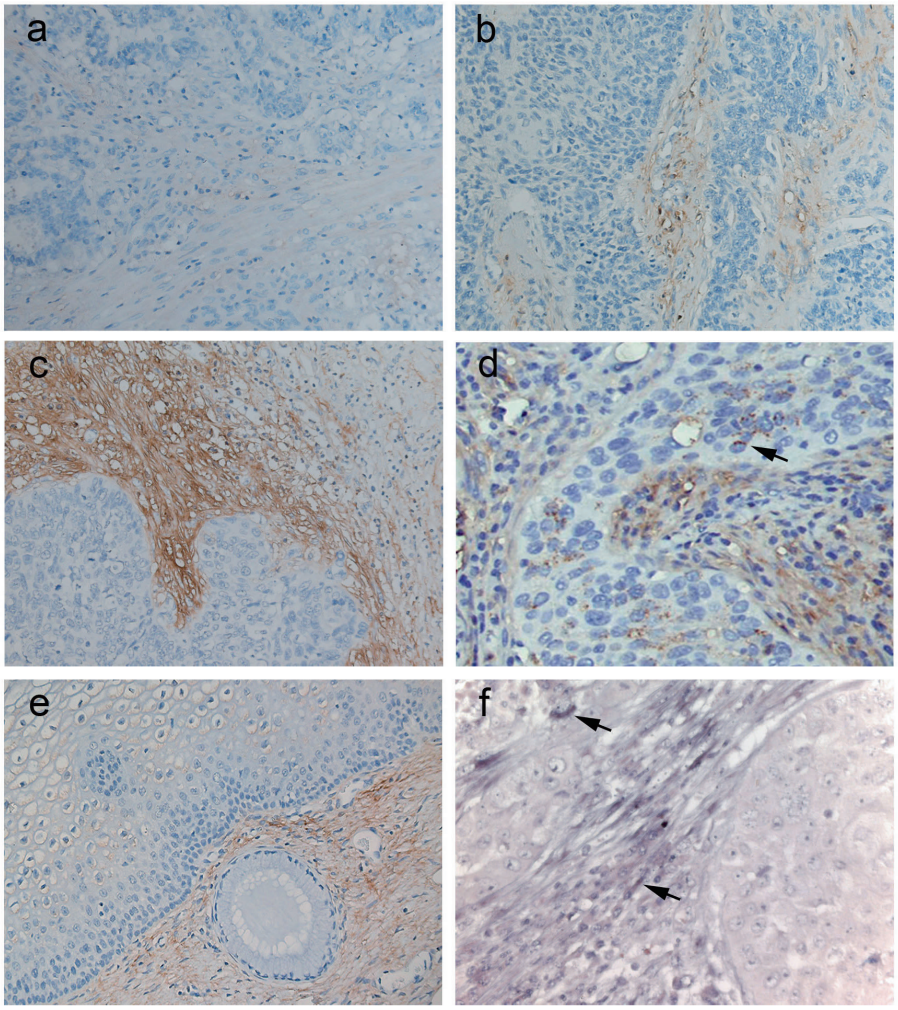


Figure 1. The expression and location of versican.
The expression and location of versican was determined using immunohistochemistry as well as RNA-*in situ* hybridization as described in Materials and methods (x250 magnification). (**a**) Cervical tumor, weak versican staining; (**b**) Cervical tumor, moderate stromal versican staining; (**c**) Cervical tumor, strong stromal versican staining; and (**d**) Cervical tumor, strong stromal versican staining (x400 magnification). The arrow indicates a positive tumor cell. (**e**) Subepithelial versican staining in normal cervical tissue and (**f**) RNA-*in situ* hybridization cervical tumor with weak cytoplasmic staining of stromal cell clusters (magnification, x400). Arrows indicate positive stromal cells.

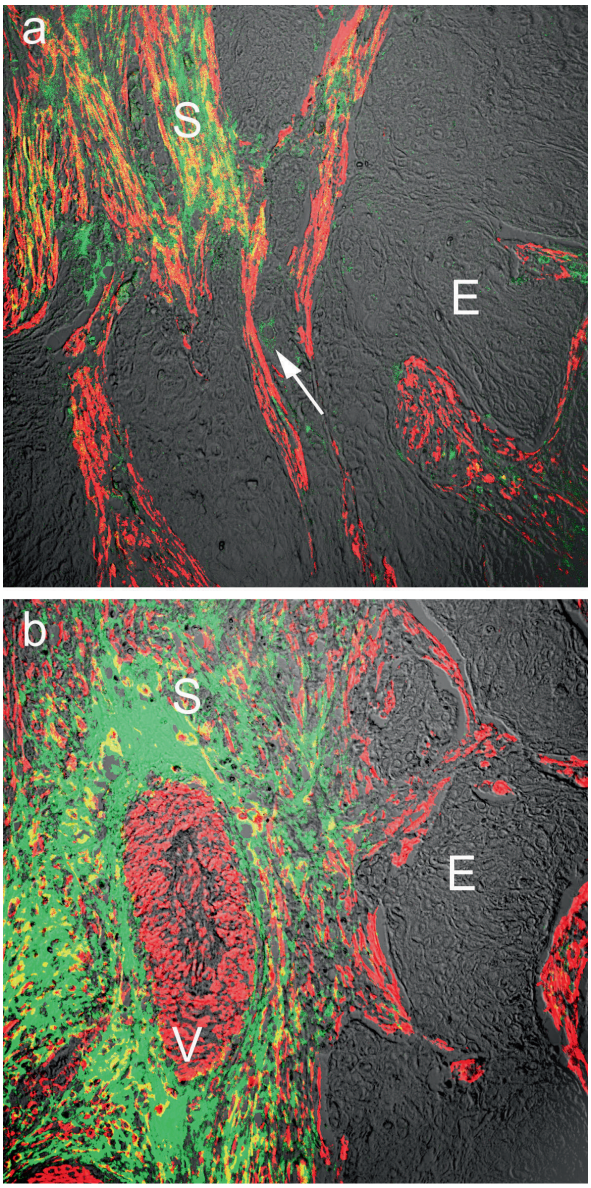


Figure 2. The expression and location of versican by stromal cells and tumor cells.
The expression and location of versican and smooth muscle actin (SMA) was determined using fluorescent immunohistochemistry as described in Materials and methods (magnification, x250). (**a**) Cervical tumor, strong stromal versican staining (green) and strong stromal SMA staining (red); the arrow indicates positive tumor cells, colocalization of versican and SMA in stromal cells (yellow). (**b**) Cervical tumor, strong stromal versican staining (green) and strong SMA staining of vessels (red). E, epithelial tumor cells; S, stroma; V, Vessel.

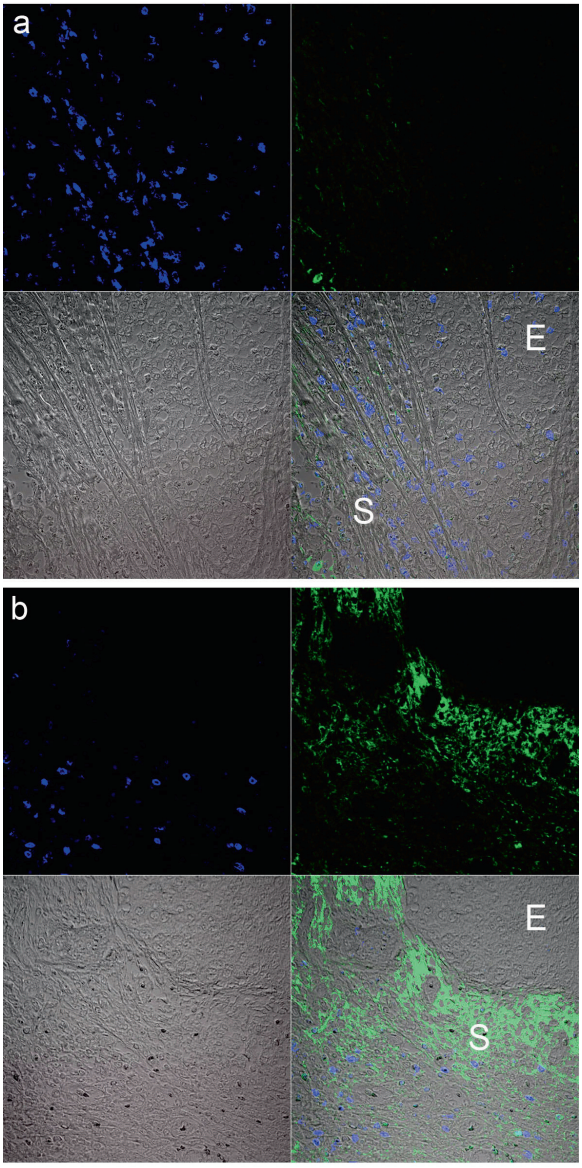


Figure 4. The expression of versican and location of CD8-positive T cells.
The expression of versican (green) and location of CD8-positive T cells was determined using fluorescent immunohistochemistry as described in Materials and methods (magnification, x250). The tumor is visualized as a phase-contrast Nomarski image (grey). (**a**) Cervical tumor, weak versican staining in the peripheral stromal area (green) was associated with a high number of intraepithelial CD8-positive T cells (blue); (**b**) Cervical tumor, strong versican staining in the peripheral stromal area (green) was associated with a low number of intraepithelial CD8-positive T cells (blue). E, epithelial tumor cells; S, stroma.