Transforming Growth Factor beta-1 in cervical cancer

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Summary and General Discussion

From the studies presented in this thesis it can be concluded that cervical carcinoma cells produce cytokines and growth factors that modulate the host immune response at the tumor site. Via a decreased production of different pro-inflammatory cytokines and the strong production of TGF-β_1, a state of local immune suppression might be achieved. The production of TGF-β_1 further augments the formation of tumor stroma, an indispensable component of solid tumors. Tumor cell derived PAI-1 and $\alpha v\beta 6$, both inducible by TGF-β_1, are demonstrated to be independent prognostic factors for worse survival. In this chapter the studies are summarized and put into perspective.

The occurrence of cervical intra epithelial neoplasms and invasive carcinomas is strongly associated with infection with high-risk human papillomaviruses (HPVs). During carcinogenesis viral DNA frequently becomes integrated in the host cell DNA.[1] Expression of the viral early proteins E6 and E7 leads to immortalisation of cervical keratinocytes,[2-5] which results in unbridled proliferation of transformed or DNA-damaged cells. Besides these and other genetic alterations, (failure of) the cellular immune response is believed to play an important role in the development of HPV related neoplasms.[6-13] As mediators of cellular immunity, cytokines are of specific interest in the evolvement of many (epithelial) cancers and cervical cancer as a virus-associated cancer in particular. The observation of diminished type 1 or/and elevated type 2 cytokine production pattern in patients with different epithelial cancers such as renal cell carcinoma, bronchogenic cell carcinoma, squamous cell carcinomas as well as in patients with HPV related carcinomas raised the question whether this mechanism might be one used by cervical tumor cells to accomplish immune evasion at the tumor site.[14-22] To investigate whether tumor cells contribute to local immune suppression by the production of immunomodulatory cytokines, we describe in **chapter 2** the cytokine expression profiles of normal cervical epithelial cells, malignantly transformed cervical epithelial cell lines and tumor cell suspensions, measured by means of RT-PCR and Southern blotting. The expression of four different groups of cytokines was investigated: pro-inflammatory, anti-inflammatory, growth factors and chemotactic factors for monocytes and eosinophils.[23,24] We demonstrated that after malignant transformation cervical epithelial cells have a decreased ability to express both Th1 and Th2 cytokines, since TNF-α, GM-CSF, IL-5 and IL-10 mRNA were less frequently expressed in carcinoma cell lines compared to normal epithelial cells. An increased ability for cytokine expression in carcinoma cell lines was only found for MCP-1 mRNA, a chemoattractant for monocytes and macrophages. The decreased expression of TNF-α and GM-CSF in tumor cells might contribute to a less effective local anti-tumor immune response, since both cytokines are involved

in augmenting antigen presentation, maturation and cytotoxicity of Langerhans cells. GM-CSF further induces production of the pro-inflammatory IL-12 by dendritic cells, a mechanism important in the activation of naïve T lymphocytes and in the generation of virus specific immune responses.[25,26] Cervical carcinoma cells apparently do not use the up regulation of immunosuppressive cytokines such as IL-5 and IL-10 to escape immunosurveillance, as these cytokines were expressed to a lesser extent in carcinoma cell lines than in normal epithelial cells. The enhanced local expression of IL-10 in precancerous lesions observed by others,[27,28] suggests that a local milieu of immunosuppression is created. This might be the effect of inflammatory cells rather than of epithelial cells, since all our tumor cell suspensions produced this cytokine in contrast to carcinoma cell lines. The presence of an eosinophil rich tumor infiltrate as regularly observed in cervical carcinomas and suggested to be indicative of a less effective immune response, could not be attributed to the an enhanced expression of eotaxin, a chemotactic factor for eosinophils by the carcinoma cells. The few carcinoma cell lines that expressed this chemotactic factor did not originate from cervical carcinomas with a majority of eosinophils in the tumor infiltrate. Possibly, an eosinophilic tumor infiltrate might be due to IL-4 production by carcinoma and/or inflammatory cells.[29] Alternatively, other cytokines not investigated in this study may be important. MCP-1 (CCL2), a chemoattractant for monocytes and macrophages, was expressed in all carcinoma cell lines in contrast to only one normal epithelium. Our data are in contrast with those of Kleine *et al*., who based on their findings, suggested that down regulation of MCP-1 is part of the program of high-risk HPV E6/E7-induced transformation of primary epithelial cells.[30] Interestingly however, Zijlmans *et al*. measuring MCP-1 expression in a cohort of cervical carcinoma patients concluded, that the absence of MCP-1 in cervical carcinoma cells was a prognostic favorable factor (unpublished data). In this study it was suggested that the enhanced MCP-1 expression in tumor cells might result in the attraction of monocytes, that subsequently differentiate into macrophages (TAM), to the tumor site. At the tumor site TAM expose less anti-tumor activities due to decreased local TNF-α and GM-CSF expression and strong TGF-β_1 production. Since these TAM form an important source of the tumor growth stimulating IL-6 and of proteases and angiogenic growth factors, the net effect might be tumor progression.[31-33] Together our data support the idea that both tumor cells and inflammatory cells contribute to the cytokine environment at the tumor site, creating a milieu of potential immunosuppression.

TGF-β_1, a cytokine and growth factor with strong anti-inflammatory properties, was demonstrated to be expressed in normal epithelial cells as well as in carcinoma cell lines and tumor cell suspensions. TGF-β_1 is secreted in an inactive form

and has to be activated before it can bind to its receptors and initiate intracellular signaling resulting in the transcription of TGF-β_1 dependent factors such as PAI-1, the inhibitor of the proteases u-PA and t-PA. To investigate whether carcinoma cell derived (active) TGF-β_1 implements its diverse functions described *in vitro* in cervical carcinoma tissue *in vivo*, we determined the expression of TGF-β_1 mRNA in tissue specimens of a cohort of cervical cancer patients, which is described in **chapter 3**. To verify biological activity of TGF-β_1 we examined the expression of PAI-1 protein in the tissue specimens. We demonstrated a significant, inverse correlation between strong TGF-β_1 expression and the presence of an inflammatory infiltrate, which illustrates the immunosuppressive properties of this cytokine at the tumor site. Since we did not investigate the composition of the inflammatory infiltrate, we do not know which type of cell from the immune system in particular is influenced by enhanced TGF-β_1 production. The inflammatory infiltrate of cervical carcinomas consists in majority of macrophages and T lymphocytes, with varying amounts of other cells such as NK cells, immature dendritic cells and neutrophilic and eosinophilic granulocytes.[23,34] TGF-β_1 expression *in vivo* has been correlated with a reduced CD8+ cell influx[35] as well as with enhanced macrophage infiltration[36] and is known to interfere with the generation of CTLs and the proliferation of T lymphocytes.[37] According to our results, the extent of the tumor infiltrate was not related to a better survival rate (data not shown), which might support the opinion that the activity of immune cells in the inflammatory infiltrate is of more importance than the number of inflammatory cells present.[34,38,39] The activity of the inflammatory cells might be influenced by the paracrine effects of TGF-β_1, as well as by the effects of decreased local TNF-α and GM-CSF. The attraction of preferentially Th2 and T regulatory lymphocytes to the tumor site might result in an extended, yet not immunosupportive infiltrate.[40,41] However, the inflammatory reaction observed in most cervical cancer specimen, may have a dual significance: on the one hand it might reflect the attempt of the host's immune system to eradicate the tumor, while on the other hand it also facilitates invasive growth of (pre-) malignant cells by basement membrane brake down, remodelling the ECM and induction of angiogenesis, through the production of proteases and angiogenic growth factors.[42,43] Since these factors are especially produced by TAM, up-regulated expression of MCP-1 by tumor cells might play an important role in this process. It is likely that the balance in the cytokine network in the tumor environment determines whether the tumor infiltrate has either a more anti-tumor or pro-tumor effect.

The expression of TGF-β_1 mRNA in tumor cells positively correlated with the amount of intratumoral stroma, which suggests that TGF-β_1 is of importance in

stroma formation in cervical carcinomas. This is in agreement with observations in mammary ductal carcinomas, desmoplastic pancreatic cancers, scirrhous gastric carcinoma and some rare types of thyroid papillary carcinoma, where also tumor cell, not stroma cell, derived TGF-β_1 was associated with a more extensive formation of tumor stroma.[44-47] This might be (partly) explained by the chemotactic effect of TGF-β_1 on fibroblasts and its growth stimulating properties on fibroblasts. The tumor stroma provides the vascular supply that tumors require for nourishment, gas exchange and waste disposal and is thought to indispensable for the growth of solid tumors.[48] In addition, recent data support the idea of a role for the tumor stromal environment as a leading player, and not just a supporting extra, in the initiation of carcinomas, since intracellular cross-talk may occur within tissues via the production of paracrine growth factors.[49] Excessive formation of tumor stroma has been associated with a more aggressive growth pattern as well as with inhibition of lethality.[46,50,51] We found however no correlation between the amount of intratumoral stroma and prognostic unfavorable parameters (data not shown).
The composition of the tumor stroma nevertheless may also be important. Strong TGF-β_1 mRNA expression by tumor cells correlated with more collagen deposition in the tumor stroma. Since the mechanical quality of the extra cellular matrix is mainly determined by the properties of its collagenous component, this raised the question whether this small subgroup with a desmoplastic tumor stroma would be more effective in protecting the tumor cells from the host immunological defence mechanism. Indeed we observed in this subgroup a trend towards the presence of a less extensive inflammatory infiltrate in the tumor (data not shown). In agreement with our former observations on the tumor infiltrate, this did not result in more aggressive tumor growth (data not shown).

Since the elevated expression of TGF-β_1 is associated with a worse survival in many different types of cancer we describe in **chapter 4** the correlation between TGF-β_1 expression in tumor cells and clinicopathological parameters known to be of importance in cervical cancer, as well as its prognostic relevance regarding (disease free) survival. The expression of TGF-β_1 in carcinoma cells did not correlate with any of the investigated parameters (other than tumor stroma, extent of inflammatory infiltrate and collagen deposition), nor was it predictive for disease free survival. Different studies have demonstrated that the expression of TGF-β_1 by mainly squamous cervical epithelial cells decreased during malignant transformation from normal cervical epithelium via CIN to invasive carcinoma.[52-55] The serum levels of TGF-β_1 in carcinoma patients compared to CIN patients or normal individuals were demonstrated to be lower in carcinoma patients,[56,57]

although one study showed that (higher) pre-treatment TGF-β_1 plasma levels were predictive for a worse disease free survival.[58] In most other types of cancer such as gastric carcinoma, breast carcinoma, colon and prostate carcinoma, enhanced TGF-β_1 production correlates with more advanced disease stage, depth of infiltration and shorter survival rates, which is thought partly to be the effect of inducing angiogenesis, direct of via VEGF induction, ECM remodeling and local immune suppression.[59-62] Bladder cancer is the only type of cancer in which, comparable to cervical squamous cell carcinoma, a loss of expression of TGF-β_1 comparing late stage to early stage disease was observed.[63] Apparently, the role of TGF-β_1 in several types of cancer might differ. These observations, together with the lack of correlation between TGF-β_1 over expression and important prognostic parameters for progressive disease such as infiltration depth and lymph node metastasis observed in our study group, might suggest, that in cervical cancer the loss of TGF-β_1 regulated growth restriction might be of importance early in carcinogenesis. In a later stage over expression might induce biological effects such as increased stroma formation and decreased tumor infiltrate thus optimizing the biological surroundings of the tumor cells. Such biphasic effects of TGF-β during tumorigenesis have been proposed by others as well.[64,65] TGF-β_1 and several components in the TGF-β-SMAD signaling system such as TGF-RI and TGF–RII, SMAD 2 and SMAD 4, might initially act as tumor suppressors since they prevent the unbridled proliferation of DNA damaged cells. Tumor cells might escape this negative growth regulation by producing less autocrine TGF-β_1 [66] or by mutations in one of the signaling pathway components, as have been described in cervical carcinoma, as well as in other malignancies.[67-71]

We observed that TGF-β_1 was expressed more often in adeno (-squamous) carcinomas than in squamous cell carcinomas, which is in agreement with observations by Santin *et al.*[72] In contrast to the described decrease of TGF-β_1 expression in most squamous cell carcinomas, in adenocarcinomas an increase was detected during malignant transformation from endocervical epithelium to adenocarcinoma.[73] Most of the other malignancies described above, in which over expression was related to an unfavorable prognosis, are adenocarcinomas as well. Together these data suggest a possible different role for TGF-β_1 in adenocarcinomas than in squamous cell carcinomas.

An additional explanation for the observed lack of correlation between autocrine TGF-β_1 and prognostic unfavorable factors might be, that the amount of TGF-β_1 mRNA present in the carcinoma cells scored by us does not proportionally correlate with the amount of active TGF-β_1 protein present in the tumor cells. PAI-1 protein expression was determined in parallel with TGF-β_1 expression to verify biological activity of TGF-β_1, since active TGF-β_1 is known to induce the

transcription of the PAI-1 gene *in vitro* and *in vivo* dose-dependently, even if cells have become insensitive to other TGF-β_1 regulated functions such as growth inhibition.[74-78] Co expression of both factors was observed in all tumors, although not quantitatively correlated, which might be the result of comparing mRNA expression of TGF-β_1 with protein expression of PAI-1. This suggests that at least part of the TGF-β_1 mRNA results in transcription of active protein, but if the amount of TGF-β_1 mRNA observed approximately reflects the amount of active TGF-β_1 protein present, remains the question. Surprisingly, the presence of PAI-1 protein in cervical tumor cells was demonstrated to be an independent prognostic unfavorable parameter and correlated significantly with a higher FIGO stage and the presence of metastases. This is in agreement with other studies on PAI-1 in cervical carcinoma[79,80] as well as in other types of cancer.[81-86] The correlation between PAI-1 expression and tumor aggressiveness in many cancers is still poorly understood, as its main functions are inhibition of plasmin regulated proteolysis and regulating cell adhesion and detachment from ECM components such as vitronectin.[87] As hypothesized by others, a possible explanation might be that via autocrine PAI-1 production the tumor protects itself against proteolytic degradation, which the tumor imposes on the surrounding normal tissue. At the same time PAI-1 might effectuate paracrine functions such as inducing angiogenesis,[79,82,88] inasmuch as absence of PAI-1 in mice has been demonstrated to inhibit angiogenesis. Additionally, PAI-1 might stabilize the matrix scaffold required for tumor cell and endothelial cell migration and the assembly of endothelial cells into capillaries, as excessive degradation of extra cellular matrix is incompatible with efficient cellular migration.[89,90]

Latent TGF-β can be activated by the αvβ6 integrin and active TGF-β is as a key inducer of EMT *in vitro*.[91,92] In **chapter 5** we examined the expression of the αvβ6 integrin in normal epithelium, CIN, primary squamous cell carcinomas and lymph node metastases and the mutual correlations between expression in cancer cells and TGF-β_1 expression, the ECM substrate fibronectin and other clinicopathological parameters. Local dissemination and intravasation of malignant cells requires a more motile phenotype of the cells, which is called EMT.[93] The αvβ6 integrin is hardly expressed on normal epithelia, but up regulated in different processes of enhanced cell migration such as wound healing, placentation and carcinomas of different origin. The increased expression of αvβ6 in carcinoma cells is thought to be a consequence of EMT.[94] Indeed we observed a gradual up regulation of αvβ6 integrin from normal epithelium via CIN towards invasive carcinomas, while among carcinomas stronger presence of αvβ6 in carcinoma cells correlated with advanced disease and worse overall and disease free survival. The presence of TGF-β and αvβ6 were correlated with each other *in vivo*. Induced expression

of αvβ6 in carcinomas might provide a mechanism to locally activate TGF-β function *in vivo*, provide a feedback loop to perpetuate the EMT process and in turn, provide a tumor microenvironment more amenable to progression.[94] Besides for LAP of TGF-β, the αvβ6 integrin is a high affinity receptor for fibronectin, which is illustrated by the enhanced motility of αvβ6 expressing carcinoma cells on a fibronectin rich matrix.[94,95] The abundant presence of this ECM protein in the tumor stroma of cervical carcinomas which we described in **chapter two**, might thus provide an excellent pathway for migration of αvβ6 expressing carcinoma cells and facilitate invasion. Additionally, the observed high expression of PAI-1 in some carcinomas might even attribute in directing the tumor cells towards fibronectin, since a recent study by Isogai *et al.* demonstrated that PAI-1 expression in endothelial cells stimulates endothelial cell migration towards fibronectin by competitively binding to vitronectin.[96] Contradictory to this idea however, other investigators have reported on the anti-migratory properties of PAI-1 and question the importance of PAI-vitronectin binding in migration.[97,98]

Summarizing from our studies it can be concluded that cervical carcinoma cells can modulate their environment. The balance in the cytokine environment of a tumor may determine whether the accompanying inflammatory reaction is more anti-tumorigenic or pro-tumorigenic. When developing immunological strategies that also need to perform their anti-tumor activities locally at the tumor site, it is important to reckon with this immunosubversive behaviour that tumor cells can display. Methods such as transduction of tumor cells with genes encoding for GM-CSF, IL-2 or IL-12, has been demonstrated to be feasible in mice, and might restore part of the local anti-tumor immune reaction. Furthermore, we hypothesize, that during cervical squamous cell carcinogenesis, (pre-) malignant cells might use down regulation of autocrine TGF-β_1 to escape from control of cell proliferation. In cervical adenocarcinomas a different mechanism might occur. The effects of tumor cell derived TGF-β_1, both on the tumor stroma as on the tumor cells - as observed by the induction of PAI-1 and αvβ6 production -, appear to create an environment in which tumor growth and invasion is augmented. In future clinical trials, especially in the field of immunotherapy, the presence of certain cytokines and integrins, such as PAI-1 and αvβ6, should be taken into account.

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Chapter 7