Transforming Growth Factor beta-1 in cervical cancer

Hazelbag, S.

PROGNOSTIC RELEVANCE OF TGF-β_1 AND PAI-1 IN CERVICAL CANCER

Suzanne Hazelbag[1,2], Gemma G. Kenter[2], Arko Gorter[1] and Gert Jan Fleuren[1],

[1] Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Obstetrics and Gynaecology, Leiden University Medical Center, Leiden, the Netherlands

International Journal of Cancer 112: 1020-1028, 2004

Abstract

Objective: cervical carcinoma is a HPV related, immunogenic type of malignancy, in which escape of the tumor from the hosts' immune response is thought to play an important role in carcinogenesis. The multifunctional cytokine Transforming Growth Factor-β_1 (TGF-β_1) is involved in immunosuppression, stroma and ECM formation and controlling (epithelial) cell growth. The plasminogen activating (PA) system plays a key role in the cascade of tumor-associated proteolysis leading to extracellular matrix degradation and stromal invasion. Changes in expression of components of this system, including Plasminogen Activator Inhibitor-1 (PAI-1), have been associated with poor prognosis in a variety of solid tumors. The present study was undertaken to assess the role of both components on relapse, survival and other clinicopathological parameters in cervical cancer.

Methods: the expression of TGF-β_1 mRNA in 108 paraffin embedded cervical carcinomas was detected by mRNA *in situ* hybridization. Immunohistochemistry was used to investigate the expression of PAI-1 protein.

Results: the presence of cytoplasmatic TGF-β_1 mRNA in tumor cells was not significantly correlated with the other clinicopathological parameters investigated nor with a worse (disease free) survival. Expression of the PAI-1 protein in tumor cells was strongly correlated with worse overall and disease free survival, in addition to well known prognostic parameters like lymph node metastasis, depth of tumor infiltration, tumor size and vasoinvasion. In the multivariate analysis PAI-1 turned out to be an independent, strong prognostic factor. In a subgroup of patients without lymph node metastases, PAI-1 was predictive for worse survival and relapse of disease, too.

Conclusions: our results show that the enhanced expression of PAI-1 by carcinoma cells is correlated with worse (overall and disease free) survival of patients with cancer of the uterine cervix. The expression of TGF-β_1 in itself is not associated with worse survival in these patients. Although simultaneous presence of the two factors was observed in all tumors, induction of PAI-1 by TGF-β_1 could not be demonstrated in our group of cervical carcinomas.

Introduction

Cervical cancer is the second leading cause of cancer death in women worldwide.[1] It is well established that chronic infection of keratinocytes with high risk human papilloma viruses (HPV) plays an important role in the pathogenesis of cervical cancer.[2] The host cellular immune response is thought to be essential in controlling both HPV infections and HPV- related neoplasms.[3-6] In previous studies we have shown that cervical carcinoma cells are capable of producing the multifunctional cytokine TGF-β_1 and that enhanced production is associated with a diminution of the tumor infiltrate and an increase in the amount of intra tumoral stroma in a group of patients with cancer of the uterine cervix.[7,8] Besides in immunosuppression, this cytokine is in most cells involved in controlling cell proliferation by exerting a growth-inhibitory activity via a reversible G1 arrest.[9,10] Furthermore, TGF-β_1 induces angiogenesis, the formation of stroma and the deposition of extra cellular matrix (ECM) by stimulating fibroblasts and other cells to produce ECM proteins like collagens, fibronectin, vitronectin, laminin and proteoglycans.[8,11,12] Concomitantly, TGF-β_1 down-regulates the expression of ECM-degrading proteases and induces proteinase inhibitors like tissue inhibitor of metalloproteinase (TIMP) –1 and PAI-1.[13-15]

PAI-1 is an important physiological regulator of ECM homeostasis and cell motility and induces angiogenesis. By inhibiting the activities of the serine proteases urokinase-type and tissue-type plasminogen activators (u-PA and t-PA) it controls the generation of plasmin out of plasminogen. PAI-1 mediates cell adhesion and cell detachment to ECM components like vitronectin. Although PAI-1 is thought to protect the ECM from degradation by proteases, a crucial step for cancer invasion, is the (enhanced) expression of this factor in many cancers associated with a worse survival rate.[16,17] *In vitro*, PAI-1 is strongly and dose-dependantly induced by biologically active TGF-β_1.[14, 18-21] Although PAI-1 expression *in vivo* might be affected by several factors, a similar correlation between TGF-β_1 and PAI-1 in different types of tissue samples has been demonstrated by several authors.[22-25] Together PAI-1 and TGF-β_1 may play an important role in the control of cell growth, tissue remodelling, angiogenesis and cancer cell invasion.[17,19]

Overproduction of both factors has been associated with poor survival in a large variety of cancers.[26-38] Data on the role of these two factors in cervical carcinogenesis are conflicting. Increases as well as decreases in TGF-β_1 expression have been reported during malignant transformation. Studies on the significance of TGF-β_1 as a clinical prognostic parameter for survival and relapse or the relationship with other established prognostic parameters contradict each other too.[39-46, 48] Although there seems to be an association between increasing PAI-1 expression

and malignant transformation of cervical epithelium, literature on the prognostic consequence of PAI-1 in cervical cancer is scarce and conflicting.[38,47]
The goal of our study was to examine the significance of both factors for the clinical outcome of patients with cervical carcinoma. In a group of patients with comparable stages of disease we analysed the relationship of TGF-β_1 and PAI-1 presence in carcinoma cells with well established prognostic parameters such as FIGO stage, lymph node metastases, vasoinvasion, depth of infiltration and HPV status. Furthermore, the potential of both factors to predict survival rates and relapse of disease was evaluated.

Materials and Methods

Tissue samples
From 108 patients with carcinomas of the uterine cervix who underwent radical hysterectomy with lymphadenectomy between 1985 and 1995, formalin-fixed paraffin-embedded tissue blocks were retrieved from the archives of the Department of Pathology, Leiden University Medical Center. None of the patients had received any therapy prior to surgery. For immunohistochemistry, paraffin blocks containing a representative part of the tumor were used.

Histopathological and clinical features
Slides of all tumors were reviewed using conventional histologic sections stained with hematoxylin and eosin.[49] Tumors were classified as squamous cell carcinoma, adenocarcinoma or adenosquamous carcinoma. Periodic acid-Schiff staining with diastase pretreatment and Alcian-blue staining was used to assign tumors with mucin production and squamous morphology to the adenosquamous category. By reviewing the slides the following data were obtained: tumor size, grade of differentiation, presence of vascular invasion, depth of tumor infiltration expressed in millimeters of tumor at right angle to the basement membrane, presence of tumor positive resection margins, parametrial involvement and lymph node involvement. The number of positive lymph nodes was specified. The size of the primary tumor was subdivided into categories of < 30 mm and > 30 mm; a second subdivision was made into < 40 mm and > 40 mm. Presence of vascular invasion was recorded. The depth of infiltration of the tumor was classed as < 15 mm and > 15 mm.
The records of all women surgically treated for carcinoma of the uterine cervix at our hospital during the period of 1985 until 1995 were reviewed. The mean age of the patients was 45,4 years, with the youngest patient 26 and the oldest

80 years at time of the surgery. A radical hysterectomy type III with pelvic lymph node dissection had been performed by three gynecologic oncologists of the department of gynecology. The following data were collected for analysis from patients records: FIGO stage, tumor size, presence of distant metastases and whether or not postoperative radiotherapy was performed. Follow-up of patients until 2001 gave information concerning recurrence free interval and performance status at last time of follow-up.

RNA in situ hybridization

RNA *in situ* hybridization was performed on paraffin-embedded sections of the 108 cervical tumors and carried out as previously described.[50,51] In short, we used a *Sma*I-*Bam*HI fragment of TGF-β_1 complementary DNA (cDNA) cloned into pBluescript KS (Stratagene, La Jolla, CA). The specific copy RNA (cRNA) probes were labeled with digoxigenin following the manufacturer's protocol (Boehringer, Mannheim, Germany). After pretreatment the tumor sections were hybridized with 10 ng TGF-β_1 antisense riboprobe per slide for 16 h at 62 ^{0}C. Subsequently, sections were washed in 2x standard saline solution citrate (SSC) with 50% formamide at 50 ^{0}C, then in 0.1x SSC with 20 mM β-mercaptoethanol at 62 ^{0}C, and finally treated with 2 U/ml ribonuclease (RNAse) T1 (Roche, Basel, Switzerland) in 2x SSC plus 1 mM ethylenediaminetetraacetic acid (EDTA) at 37 ^{0}C. Immunodetection of digoxigenin-labeled hybrids was done using nitro blue tetrazolium (NBT) as chromogen and bicholylindolyl phosphate (BCIP) as coupling agent (Roche). Blue staining of the cytoplasm indicated positivity for TGF-β_1 mRNA. To determine the level of nonspecific binding, adjacent tumor slides were hybridized with TGF-β_1 sense riboprobes. These were included as negative controls and did not show staining. Normal kidney tissue served as a positive control. The specificity of the TGF-β_1 probe has been thoroughly tested in our lab. The probe was sequenced to verify its sequence and by Northern blotting one specific band of the appropriate size was demonstrated. They detected high expression of TGF-β_1 mRNA in the distal tubuli of the kidney which was confirmed by quantitative real time PCR.[50,51]

Immunohistochemistry

Immunohistochemistry on the whole series in one experiment was performed on 4 μm sections using aminopropylethoxysilane (APES) coated slides. Paraffin sections were deparaffinized and rehydrated, and endogenous peroxidase was quenched with 0.3 % H_2O_2 in methanol for 20 min. Monoclonal antibodies directed against PAI-1 were used in a 1:500 solution and supplied by American Diagnostics Greenwich, CT. Phosphate buffered saline (PBS) containing 1% bovine serum albu-

mine (BSA) was used as diluent for the antibodies. Incubations were performed at room temperature. Washing in between incubations was performed 3 times for 5 min each in PBS. After washing in PBS, slides were incubated overnight with the specific primary antibodies. Biotin-labeled rabbit anti mouse immunoglobulins and a biotinylated horseradish peroxidase (HRP)-Streptavidin complex (both DAKO) were subsequently applied for 30 min each. To visualize immune complexes a 0.05% solution of diaminobenzidine (Sigma) containing 0.0018% H_2O_2 in a 0.05 M Tris-HCl buffer (pH 7.6) was applied. Mayer's Hematoxylin was used for counterstaining of the slides.
Brown staining of cytoplasm indicated positivity for PAI-1 in tumor cells. Brownstaining of ECM components indicated positivity for PAI-1 in stroma. Mouse IgG1 (10 μg/ml) and PBS/BSA 1% on serial slides were used as a negative control. A breast carcinoma was used as positive control and sections were stained simultaneously.

Immunohistochemical evaluation
Staining for TGF-β_1 mRNA and PAI-1 protein in tumor cells was scored semiquantitatively according to a system proposed by Ruiter *et al.*[52] The slides were independently scored by two of us. When slides were scored differently by the two observers, which occurred in a few cases, they were evaluated again by the two observers simultaneously until consensus was reached. Scores representing the percentage of tumor cells stained positive were as follows: 0 (no positive tumor cells); 1 (1-5%); 2 (6-25%); 3 (26-50%); 4 (51-75%); and 5 (76-100%). Intensity of tumor cell staining was scored as 0 (no staining); 1 (+, weak); 2 (++, clear); and 3 (+++, bright). A final, total, score was calculated by adding the scores for percentage and intensity, resulting in scores of 0 to 8. A total score of 0 was deemed negative; 2-4 was considered weak, 5-6 was considered moderate and 7-8 was considered strong.

PAI-1 staining of the tumor stroma was scored at the tumor-stromal border as sporadic, local or diffuse.[52]

HPV detection and typing
All 108 samples were tested and subtypes were determined as described before.[49]

Statistics
Statistical analysis was performed using the SPSS 11.0 software package. Associations between TGF-β_1 mRNA expression and PAI-1 expression and clinicopathological parameters were evaluated using the Fisher Exact test, and where

appropriate the Chi-squared test. Univariate analysis of overall 5 year and disease free 5 year survival was performed using the Kaplan-Meier method, the log rank test and the Cox proportional hazard models. The latter was also used for multivariate analysis. All tests were two-sided and the significance level was set to 5 %, corresponding to 95 % confidence intervals (CI).

Results

Assessment of the slides

TGF-β_1 mRNA expression was examined in the cytoplasm of cervical carcinoma cells. As a quality control for the integrity of the mRNA samples were excluded in which inflammatory cells, known to produce TGF-β_1 as well, did not stain positively (9 cases) or in which the RNA *in situ* hybridization with the household gene β-actin showed no signal (2 cases). In these cases (11 out of 108) it was assumed that the RNA quality of the specific tumor was decreased, probably due to too long fixation in the formalin. Of the remaining 97 tumors (85 squamous carcinomas, 6 adenocarcinomas and 6 adenosquamous carcinomas), total TGF-β_1 mRNA expression (i.e. sum of percentage positive cells and intensity of staining) in malignant epithelial cells was weak (score 0,2-4) in 11 cases, moderate (score 5-6) in 38 cases and strong (score 7-8) in 48 cases. Normal cervical epithelial cells, present in the majority of the tissue slides, demonstrated moderate to strong staining, as did the inflammatory cells in the tumor infiltrate.

Total PAI-1 staining ((i.e. sum of percentage positive cells and intensity of staining) of the malignant epithelial cells was weak (score 0,2-4) in 4 cases, moderate (score 5-6) in 30 cases and strong (score 7-8) in 62 cases. Subdivision of the total score into the percentage of PAI-1 positive cells and the intensity of staining showed the following results: one case no PAI-1 positive tumor cells, 3 cases 26-50% PAI-1 positive tumor cells, 8 cases 51-75% tumor positive tumor cells and 84 cases 76-100% PAI-1 positive tumor cells; one case no staining of the tumor cells, 30 cases weak (1+) staining intensity of the tumor cells, 52 cases clear (2+) intensity of the tumor cells and 13 cases bright (3+) intensity of the tumor cells. Normal cervical epithelial cells and inflammatory cells, especially eosinophilic granulocytes, showed positive staining for PAI-1 as well.

PAI-1 staining of the stroma at the tumor-stromal border was sporadic in 39 cases, local in 36 cases and diffuse in 21 cases.

Patients
Adjuvant radiotherapy was given either when lymph nodes, parametria or resection margins were tumor positive or when there was a combination of 2 or more of the following unfavorable prognostic factors: depth of infiltration ≥ 15mm, tumor size ≥ 40 mm and vasoinvasion. Of the total group of 99 patients, 3 patients were diagnosed as FIGO stage IA2, 60 as stage IB1, 12 as stage IB2 and 24 as stage II. 48 patients received postoperative radiotherapy and 30 patients suffered recurrent disease. By 2001, the authors' cut off date for follow-up, 69 patients were alive, 23 patients had died of disease, 7 patients suffered from a recurrence and 7 patients had died of causes unrelated to the primary disease and showed no evidence of disease as recorded in the clinical chart.
The mean time of disease free survival was 66 months with a minimum and maximum time of 0 and 184 months. The mean time of follow-up was 76 months ranging from 0 to 185 months. 13 patients were lost for follow-up

Association of the staining results with clinicopathological parameters
Strong expression of total TGF-β_1 mRNA (score 7 or 8) and strong staining of total PAI-1 protein (score 7 or 8) were correlated with FIGO stage, presence of tumor positive lymph nodes, distant metastases, recurrent disease, tumor size, presence of vasoinvasion, depth of infiltration, presence of tumor in the parametria, presence of tumor in the surgical margins, histology and HPV status. Table 1 shows the results of the correlation of total TGF-β_1 mRNA expression and total PAI-1 protein staining with these clinicopathological parameters. No statistically significant correlations could be detected between TGF-β_1 mRNA expression and any of the other clinicopathological parameters, although there was a trend towards relationship between strong TGF-β_1 mRNA expression and the adeno (-squamous) histologic tumor type (46% vs 75%, p 0.06).

Strong total PAI-1 staining statistically significantly correlated with FIGO stage (p 0.04), distant metastases (p 0.01), recurrent disease (p 0.01) and the squamous cell carcinoma histologic type (p 0.03). A positive correlation between TGF-β_1 mRNA expression and PAI-1 protein staining in tumor cells was observed (Fig.1). No TGF-β_1 positive /PAI-1 negative of TGF-β_1 negative/PAI-1 positive tumors were detected. However, semiquantitatively, the amount of TGF-β_1 and PAI-1 expressed in tumor cells did not demonstrate a statistically significant relationship.

Association of the staining results and clinicopathological parameters with overall and disease free survival
The survival curves for overall and disease free survival were calculated accord-

Table 1 - Association of strong TGF-β_1 expression and strong PAI-1 staining with clinicopathological parameters.

		TGF-β_1 strong			PAI-1 strong	
	n	n (%)	p-value	n	n (%)	p-value
Total	97	48		96	62	
FIGO stage						
IA + IB1	62	30 (48)	0.95	60	33 (55)	**0.04**
IB2	12	6 (50)		12	10 (83)	
II	23	12 (52)		24	19 (79)	
Lymph node						
positive	26	14 (54)	0.60	27	18 (67)	0.79
negative	71	34 (48)		69	44 (64)	
Metastases						
present	23*	12 (53)	0.72	23*	20 (87)	**0.01**
not present	73	35 (48)		72	41 (57)	
Recurrent disease						
yes	29*	15 (52)	0.72	30*	25 (83)	**0.01**
no	67	32 (48)		65	36 (55)	
Tumor size						
< 30 mm	40*	21 (53)	0.67	38*	27 (71)	0.26
≥ 30 mm	52	25 (48)		52	31 (60)	
Vasoinvasion						
present	52*	30 (58)	0.08	53*	38 (72)	0.12
not present	43	17 (40)		41	23 (56)	
Depth of infiltration						
< 15 mm	63*	30 (48)	0.96	62*	37 (60)	0.19
≥ 15 mm	27	13 (48)		27	20 (74)	
Parametia						
tumor positive	16*	7 (44)	0.65	17*	11 (65)	0.96
tumor negative	80	40 (50)		78	51 (65)	
Surgical margins						
tumor positive	20*	12 (60)	0.32	20*	16 (80)	0.10
tumor negative	76	36 (47)		75	45 (60)	
Histology						
squamous	85	39 (48)	0.14	83	57 (69)	**0.02**
adenosquamous	6	5 (83)		6	4 (67)	
adeno	6	4 (67)		7	1 (14)	
Histology						
squamous	85	39 (46)	0.06	83	57 (69)	**0.03**
adeno (-squamous)	12	9 (75)		13	5 (38)	
HPV						
negative	10*	3 (30)	0.08	9*	7 (78)	0.54
16	62	28 (45)		62	38 (61)	
other	22	15 (68)		23	16 (70)	
PAI-1 tumor cells						
weak/moderate	33*	18 (55)	0.62			
strong	61	30 (49)				

In case of statistical significant correlations, p-values are bold.

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

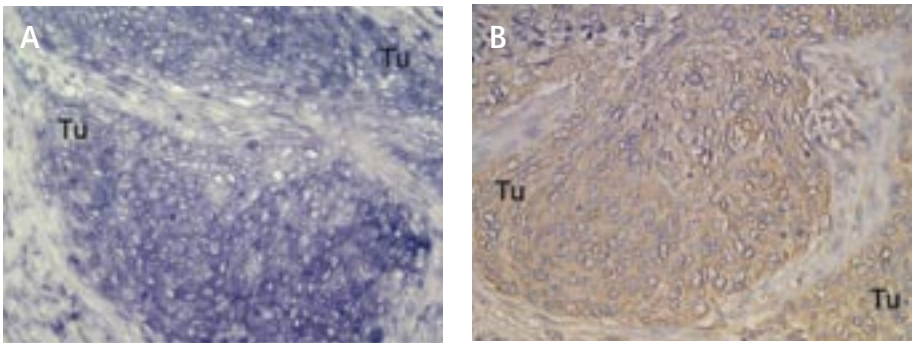

Figure 1 – TGF-β_1 mRNA expression in cervical tumor cells and corresponding PAI-1 protein expression in the same tumor. mRNA was detected by RNA in situ hybridization with an anti-sense riboprobe for TGF-β_1 and PAI-1 protein was detected by immunohistochemistry as described in material and methods. (Magnification: x 200).

A. Expression of TGF-β_1 mRNA in the cytoplasm of cervical tumor cells (Tu). TGF-β_1 is visualized by a blue color.
B. Staining of PAI-1 protein in the cytoplasm of cervical tumor cells (Tu). PAI-1 expression is visualized by a brown color.

ing to Kaplan Meier and results are shown in Table 2. The assumed proportional Hazard Risks with confidence intervals are listed in Table 3, calculated by the univariate model of Cox's regression model. As expected, the presence of tumor positive lymph nodes ($p<0.001$) (Fig.2) and a depth of infiltration ≥ 15 mm ($p<0.001$) were highly significant predictors for a shorter duration of disease free and overall 5 year survival. The presence of vasoinvasion (p 0.02), a tumor size > 30 mm (p 0.01) and FIGO stage IB2 and higher (p 0.05) also were significant predictors for both. When the cut off point for tumor size was chosen at 40 mm, associations with (disease free) survival were weakened. Patients with the adenosquamous carcinoma histologic type had a remarkable shorter 5 year overall survival (40%) vs squamous carcinoma (78%) and adenocarcinoma (100%)(p 0.04), but the groups were quite diverging in size. The presence of strong TGF-β_1 mRNA expression by tumor cells was not predictive for a worse overall or disease free survival (p 0.31/p 0.26). However, patients with strong staining for PAI-1 protein did have a worse overall and disease free survival (p 0.01/ p 0.01) (Fig.3). Also the prognostic value of the staining intensity and the percentage of positive cells of which the total PAI-1 score is composed was evaluated. The more intense the staining of the tumor cells was, the worse the overall and disease free survival was (p 0.03/0.04). The percentage of PAI-1 positive tumor cells in itself was not statistically significantly related to worse overall and disease free survival (p 0.20/0.36), although a trend could be observed (5 yr overall survival 92% vs 76% and disease free survival 92% vs 74%). However, when combined with the intensity of staining,

Table 2 - Association of clinicopathological parameters and TGF-β_1 mRNA expression/PAI-1 protein staining with overall and disease free 5-years survival.

	n	Survival, 5 years (%)	p-value	Disease-free 5-year survival (%)	p-value
Total	99	78		77	
FIGO					
IA + IB1	63	85	0.08	85	0.08
IB2	12	51		39	
II	24	69		69	
$FIGO_2$					
IA + IB1	63	81	**0.05**	85	0.06
IB2 + II	36	64		60	
Lymph node					
positive	27	57	**<0.001**	57	**0.001**
negative	72	85		83	
Tumor size					
< 30 mm	41*	90	**0.01**	90	**0.01**
≥ 30 mm	52	68		66	
Depth of infiltration					
< 15 mm	65*	88	**<0.001**	86	**<0.001**
≥ 15 mm	27	59		59	
Vasoinvasion					
present	53*	72	**0.02**	70	**0.02**
not present	44	88		87	
Histology					
squamous	86	79	**0.04**	78	0.15
adenosquamous	6	40		40	
adeno	7	100		100	
HPV					
negative	10*	90	0.50	90	0.86
16	63	76		74	
other	24	79		79	
TGF-β_1					
weak/moderate	49	82	0.33	80	0.23
strong	48	75		75	
PAI-1 (total)					
weak/moderate	34*	93	**0.01**	93	**0.01**
strong	62	69		67	
% PAI-1-positive					
tumor cells	12	92	0.20	93	0.36
≤ 75%	84	76		68	
≥ 76%					
PAI-1 intensity					
tumor cells					
weak	31	93	**0.03**	93	**0.04**
clear/bright	65	70		68	

In case of statistical significant correlations, p-values are bold.

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

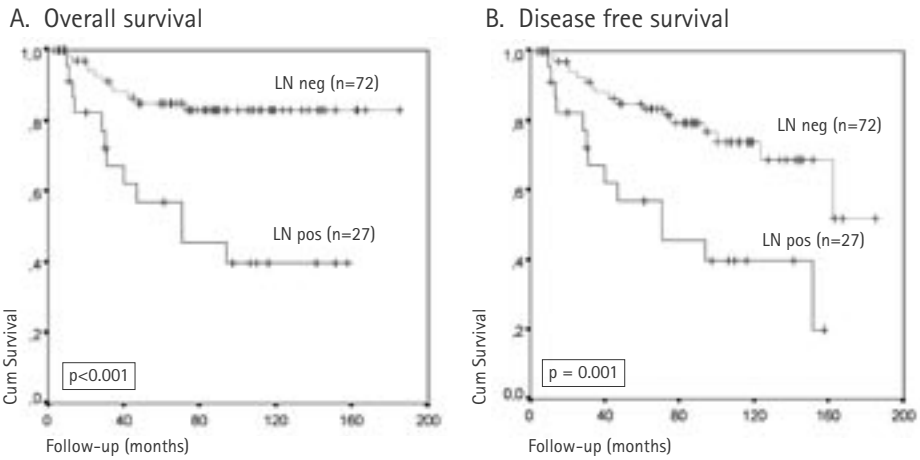

Figure 2 – Kaplan-Meier curves for (A) overall survival ($p < 0.001$) and (B) disease free survival (p 0.001) in patients with tumor positive lymph nodes (n=27) and without tumor positive lymph nodes (n=72).

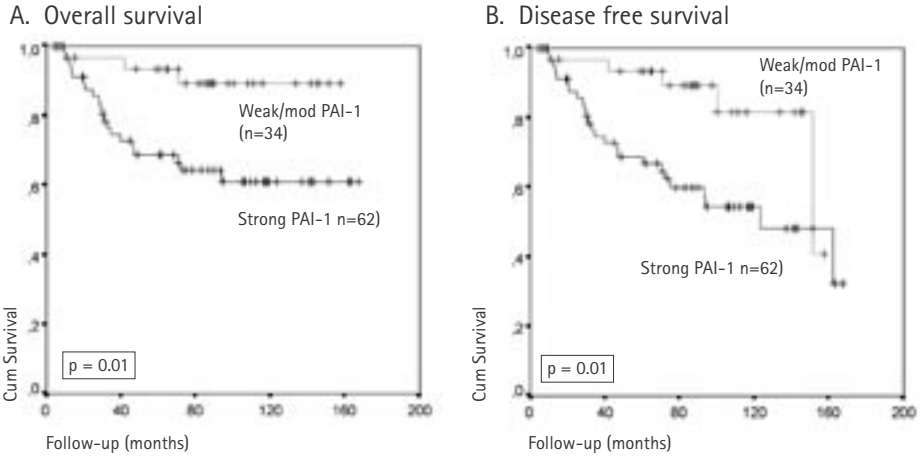

Figure 3 – Kaplan-Meier curves for (A) overall survival (p 0.01) and (B) disease free survival (p 0.01) in patients with weak/moderate PAI (n=34) and strong PAI-1 staining patterns (n=62).

Table 3 - Hazard ratio with 95% confidence interval and p- value for clinicopathological parameters and TGF-β_1 and PAI-1 staining in tumor cells as calculated by univariate analysis with Cox proportional hazard model. T.c.= tumor cells.

	Overall-survival HR (955 Cl)	p-value	Disease-free survival HR (95% Cl)	p-value
Lymph nodes				
positive	4.34 (1.91-9.87)	**< 0.001**	3.22 (1.54-6.72)	**0.002**
FIGO stage				
IA + IB1				
IB2 + II	2.24 (0.99-5.10)	**0.05**	1.96 (0.95-4.05)	0.47
Tumor size				
< 30 mm				
≥ 30 mm	3.32 (1.22-9.0)	**0.01**	2.95 (1.25-6.94)	**0.01**
Depth of infiltration				
< 15 mm				
≥ 15 mm	5.25 (2.03-13.57)	**0.001**	3.60 (1.63-7.92)	**0.002**
Vasoinvasion				
present	3.11 (1.15-8.44)	**0.03**	2.68 (1.14-6.30)	**0.02**
TGF-β_1				
weak/moderate				
strong	1.52 (0.66-3.52)	0.33	1.56 (0.75-3.27)	0.24
Total PAI-1				
weak/moderate				
strong	4.3 (1.28-14.5)	**0.02**	3.16 (1.20-8.29)	**0.02**
% PAI-1 pos. t.c.				
≤ 75 %				
≥ 76 %	3.39 (0.46-25.16)	0.23	1.93 (0.46-8.16)	0.37
Intensity PAI-1				
weak				
clear/bright	3.64 (1.08-12.26)	**0.04**	2.68 (1.02-7.04)	**0.05**

Statistical significant p-values are bold.

the percentage positive tumor cells clearly reinforced the statistical significance of total PAI-1. Subsequently, multivariate analysis was performed using the Cox's regression model on the strongest prognostic factors according to univariate analysis (Table 4) and on bothe the investigated factors (Table 5). Included in the multivariate analysis for disease free and overall survival were lymph node status, depth of infiltration, tumor size, FIGO stage and PAI-1 staining in tumor cells. PAI-1 staining was demonstrated to be an independent factor predicting worse overall (HR 8.89; p 0.04) and worse disease free (HR 4.68; p 0.02) survival, just like the depth of infiltration (HR 3.12; p 0.02 respectively HR 2.36; p 0.04) and lymph node status (HR 2.66; p 0.05 respectively HR 2.31 / p 0.06).

The staining of total PAI-1 was also evaluated in a subgroup of patients without tumor positive lymph nodes (n=69). After 5 years, the survival rate for weak/mod-

erate staining vs strong staining was 96% vs 78% (p 0.04) and the disease free survival rate 96% vs 75% (p 0.03) respectively, indicating that also in the lymph node metastasis negative group PAI-1 was a prognostic parameter for worse survival (Fig.4).

Table 4 – Hazard ratio with 95% confidence interval for strongest prognostic factors for overall and disease free survival as calculated by multivariate analysis with Cox proportional hazard model.

	Overall survival HR (95% Cl)	p-value	Disease-free survival HR (95% Cl)	p-value
Lymph node positivity	2.66 (1.01-7.0)	**0.05**	2.31 (0.998-5.44)	0.06
Total PAI-1	8.9 (1.16-67.91)	**0.04**	4.68 (1.36-16.13)	**0.02**
Depth of infiltration ≥15 mm	3.12 (1.16-8.38)	**0.02**	2.36 (1.04-5.34)	**0.04**

Statistical significant p-values are bold.

Table 5 – Hazard ratio with 95% confidence interval for PAI-1 and TGF-β for overall and disease-free survival as calculated by bivariate analysis with Cox proportional hazard model.

	Overall survival HR (95% Cl)	p-value	Disease-free survival HR (95% Cl)	p-value
PAI-1 tumor cells	3.95 (1.17-13.37)	**0.03**	2.92 (1.11-7.69)	**0.03**
TGF-β_1 tumor cells	1.37 (0.59-3.18)	0.46	1.37 (0.66-2.86)	0.40

Statistical significant p-values are bold.

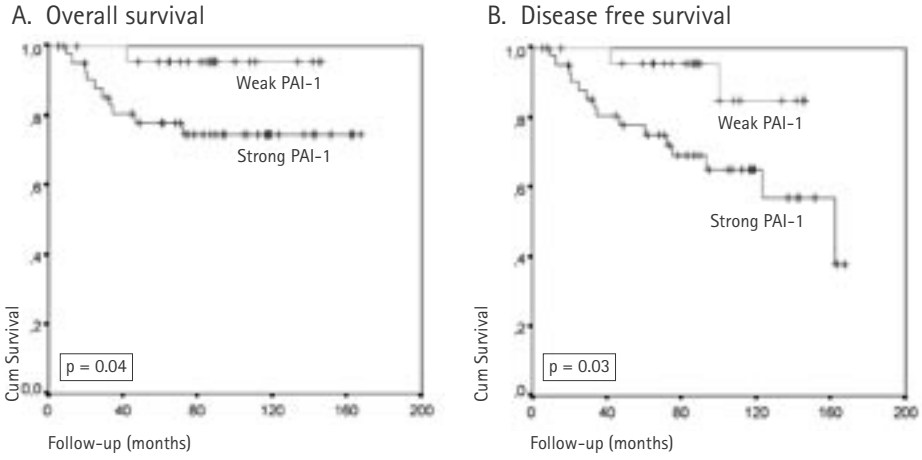

Figure 4 – Kaplan-Meier curves for (A) overall survival (p 0.04) and (B) disease free survival (p 0.03) in patients with weak/moderate PAI-1 and strong PAI-1 staining patterns in a subgroup of patients without tumor positive lymph nodes (n=72).

Discussion

We have investigated the expression of TGF-β_1 and PAI-1 by carcinoma cells in a group of patients with cervical cancer FIGO stage IA to IIB and examined the relationship with survival, recurrent disease and other clinicopathological parameters.

Our results show that strong staining of PAI-1 by cervical carcinoma cells is a significant, independent predictor for worse overall and worse disease free survival, even after including other well established prognostic factors like tumor positive lymph nodes, depth of infiltration, tumor size and vasoinvasion in the multivariate analysis. In a subgroup of patients containing lymph node metastasis negative individuals only, PAI-1 is also predictive for worse overall and disease free survival. Furthermore, we found strong PAI-1 staining by carcinoma cells to be statistically significantly related to FIGO stage, with a remarkable high percentage of strong PAI-1 staining tumors in the FIGO IB2 group, the group that also has the worst (disease free) survival rate. In addition, strong staining of PAI-1 was related to the presence of distant metastases and to recurrence of disease. According to our results, the staining of PAI-1 by stromal cells like fibroblasts was not associated with a worse (disease free) survival rate nor was it related to other clinicopathological parameters.

There are only few reports on the prognostic importance of PAI-1 in cervical cancer. Kobayashi *et al.* investigated PAI-1 expression using immunohistochemistry as well as by ELISA in the supernatant of tissue extracts. After comparing both methods the authors concluded that immunohistochemistry enabled semiquantitative determination of PAI-1 expression. In a group of 62 FIGO stage II patients these authors found the overall and progression-free survival rate to be worse in patients with strong PAI-1 staining in tumor nests compared to patients with a weak or no PAI-1 staining, which is in agreement with our findings.[47] The association they found between high PAI-1 levels and a higher percentage of lymph node metastasis could not be confirmed by us, although we detected a comparable relationship between strong PAI-1 staining and the presence of distant metastases. Because all the patients in their group were of a FIGO stage II of disease, comparisons between stages could not be made, neither was the relationship with other parameters evaluated. In another study by Horn *et al.*, PAI-1 expression in 114 cervical cancer patients (FIGO stage I to III) was evaluated and, corresponding with our study, elevated PAI-1 levels were demonstrated to be positively correlated with advanced tumor stage.[38] However, after including other prognostic parameters in their multivariate analysis, PAI-1 failed to give additional prognostic information regarding survival rates, which is in contrast

to our findings. Two studies on PAI-1 expression in CIN and invasive carcinoma versus normal epithelium reported an increase in expression during the multistep process of cervical carcinogenesis and also an increase with advancing disease stage, suggesting that this component plays a role in invasion and metastasis.[53,54] A relationship with HPV status was examined but, as in our study, not detected.
Although literature about the consequence of PAI-1 in cervical cancer is scarce, in a variety of solid tumors, i.e. breast, endometrium, stomach, colon, kidney and lung, PAI-1 has been associated with aggressiveness too and may serve as an independent prognostic factor in these diseases.[32-34,38,54,55] Some studies propose that the deposition of PAI-1 by stromal cells instead of malignant epithelial cells would be more of consequence for cancer cell invasion and angiogenesis and that tumor cells might recruit stromal cells to produce proteases thus facilitating tumor cell invasion, while others hypothesize that PAI-1 production by tumor cells serves to protect cancer tissue against the proteolytic degradation which the tumor imposes upon the surrounding normal tissue and adds to poor prognosis in that way.[56,33,47]
However, all these studies focus on the role of PAI-1 and the PA system in cancer, and do not involve the role of TGF-β_1. This multifunctional cytokine, among others, tightly regulates the production of PAI-1 and together they may cooperate in control of cell growth, tissue remodelling, angiogenesis and cancer cell invasion.[17,19] According to our results, TGF-β_1 was expressed by most tumor cells, but strong TGF-β_1 expression by tumor cells was not associated with worse survival rates, recurrent disease or any of the other clinicopathological parameters, except for histology. TGF-β_1 was more expressed by adeno and specificly adenosquamous carcinomas than by squamous carcinomas, which is in agreement with Santin *et al*.[57] In addition, Farley *et al.* reported an increase in TGF-β_1 protein and receptor expression during malignant transformation from endocervical epithelium to adenocarcinoma, which is in contrast to findings in squamous cell carcinogenesis and raises the question whether TGF-β_1 plays a different role in adeno (-squamous) carcinoma than in squamous cell carcinoma.[44] The lack of relationship between enhanced TFG-β_1 expression by (mostly squamous cell) carcinomas and poor survival we demonstrated, lends support to previous studies, which demonstrated that loss of TFG-β_1 expression was an early event in the neoplastic transformation of (squamous) cervical epithelia.[41,42,48,58,59]
However, although not directly, TGF-β_1 indirectly may affect the clinical course in cervical cancer via induction of PAI-1 in tumor cells and stromal cells. A quantitative relationship between TGF-β_1 expression and PAI-1 staining in tumor cells could not be detected, but it was notably that all TGF-β_1 positive cells produced PAI-1 too, while no TGF-negative/PAI-1 positive tumor cells were detected. Fifty-

eight out of 99 patients demonstrated local or diffuse stromal PAI-1 staining (data not shown). We hypothesize that cervical tumor cells protect themselves from degradation by PAI-1. PAI-1 may be induced by autocrine TGF-β_1 expression or acquired from the environment, which would help to explain the decreased survival rates observed in highly PAI-1 expressing tumors. Via paracrine TFG-β_1 production tumor cells are able to attract fibroblasts and to stimulate these cells to produce PAI-1, which might help facilitating invasion of cancer cells. This mechanism would suggest that the transcriptional response to TGF-β_1 remains conserved in cervical carcinomas, as is earlier demonstrated by Kang et al.[60] Whether, despite conserved transcription, the tumor cells lose their growth sensitivity to TGF-$\beta_{1,}$ as was earlier demonstrated for cervical carcinoma cells in vitro, and whether that is due to TGF-receptor mutations, downregulations or SMAD4 mutations is an interesting subject for further research.[60-66] More research on the relationship between TGF-β_1 and PAI is required in this type of cancer and especially on protein level to affirm the above proposed hypothesis.
With respect to the observation that the enhancement of TGF-β_1 in itself does not seem to influence the clinical course of cervical cancer, cervical carcinoma distinguishes it self from many other types of cancer, where elevation of TGF-β_1 production is associated with poor survival.

In conclusion, we have demonstrated co localization of TGF-β_1 and PAI-1 in cervical carcinoma cells. Enhanced presence of PAI-1 in these tumor cells appears to be a strong, independent predictor for worse overall and disease free survival and may be partly induced by autocrine TGF-$\beta_{1,}$ which expression in itself is not associated with poor survival rates. In future PAI-1 might be a helpful factor in estimating a patient's prognosis and deciding on what therapy might be best.

Acknowlegdement

The assistance of Mr. P. Eilers of the department of Medical Statistics of the LUMC in analyzing the data is gratefully acknowledged. Furthermore we thank Mr. J.J. Baelde for the kindly provided TGF-β_1 probe and Mr. H. Zijlmans for the RISH experiment with the household gene β-actin on the group of tumors.

References

1. IARC Working Group. IARC monograhs on the evaluation of carcinogenic risk to humans. 1995
2. Zur Hausen H. Papillomaviruses in human cancers. Proc Assoc Am Physicians 1999;111:581-587.
3. Wu TC. Immunology of the human papilloma virus in relation to cancer. Curr Opin Immunol 1994;6:746-754.
4. Benton C, Shahidullah H, Hunter JA. Human papillomavirus in the immunosuppressed. Papillomavirus Rep 1992;3:23-26.
5. Petry KU, Scheffel D, Bode U, Gabrysiak T, Kochel H, Kupsch E, Glaubitz M, Niesert S, Kuhnle H, Schedel I. Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. Int J Cancer 1994;57:836-840.
6. Ozsaran AA, Ates E, Dikmen Y, Zeytinoglu A, Terek C, Erhan Y, Ozacar T, Bilgic A. Evaluation of the risk of cervical intraepithelial neoplasia and human papilloma virus infection in renal transplant patients receiving immunosuppressive therapy. Eur J Gynaecol Oncol 1999;20:127-130.
7. Hazelbag S, Fleuren GJ, Baelde JJ, Schuuring E, Kenter GG, Gorter A. Cytokine profile of cervical cancer cells. Gynecol Oncol 2001;83:235-243.
8. Hazelbag S, Gorter A, Kenter GG, van den Broek L, Fleuren GJ. Transforming Growth Factor-beta 1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. Human Pathol 2002;33(12):1193-1199.
9. de Visser KE, Kast WM. Effects of TGF-β on the immune system: Implications for cancer immunotherapy. Leukemia 1999;13(8):1188-1199.
10. de Caestecker MP, Piek E, Roberts A. Role of Transforming Growth Factor-β signaling in cancer. J Natl Cancer Inst 2000;92(17):1388-1402.
11. Roberts A, Sporn M. Regulation of endothelial cell growth, architecture and matrix synthesis by TGF-β^1. Am Rev Respir Dis 1989;140:1126-1128.
12. Haralson MA. Transforming growth factor-β, other growth factors and the extracellular matrix. J Lab Clin Med 1997;130:455-458.
13. Taipale J, Saharinen J, Keski-Oja J. Extracellular matrix-associated Transforming Growth Factor-β: Role in cancer cell growth and invasion. Adv Cancer Res 1998;75:87-134.
14. Laiho M, Saksela O, Andreasen PA, Keski-Oja J. Enhanced production and extracellular deposition of the endothelial-type Plasminogen Activator Inhibitor in cultured human lung fibroblasts by Transforming Growth Factor-β. J Cell Biol 1986;103:2403-2410.
15. Allan EH, Zeheb R, Gelehrter TD, Heaton JH, Fukumoto S, Yee JA, Martin TJ. Transforming Growth Factor beta inhibits Plasminogen Activator (PA) activity and stimulates production of Urokinase-Type PA, PA Inhibitor-1 mRNA and protein in rat oseoblast-like cells. J Cell Physiol 1991;149:34-43.
16. Kutz SM, Hordines J, McKeown-Longo PJ, Higgins PJ. TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. J Cell Sci 2001;114(21):3905-3914.
17. Pappot H, Gardsvoll H, Romer J, Navrsted Pedersen A, Grondahl-Hansen J, Pyke C, Brunner N. Plasminogen Activator Inhibitor Type 1 in cancer: therapeutic and prognostic implications. Biol Chem Hoppe-Seyler 1995;376:259-267.
18. Logeart-Avramoglou D, Huynh R, Chaubet F, Sedel L, Meunier A. Interaction of specifically chemically modified dextrans with transforming growth factor β1: potentiation of its biological activity. Biochem Pharmacol 2002;63:129-137.
19. Song C, Siok TE, Gelehrter TD. Smad4/DPC4 and Smad3 mediate Transforming Growth Factor-β (TGF-β) signaling through direct binding to a novel TGF-β-responsive element in the human Plasminogen Activator Inhibitor-1 promoter. J Biol Chem 1998;273(45):29287-29290.
20. Liu C, Yao J, de Belle I, Huang R, Adamson E, Mercola D. The transcription factor EGR-1 suppresses transformation of human fibrosarcoma HT1080 cells by coordinated induction of Transforming Growth Factor-β1, fibronectin, and Plasminogen Activator Inhibitor-1. J Biol Chem 1999;274(7):4400-4411.
21. Laiho M, Saksela O, Keski-Oja J. Transforming Growth Factor-β induction of type-1 Plasminogen Activator Inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. J Biol Chem 1987;262(36):17467-17474.

22. Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, ten Dijke P, White B, Wigley FM, Goldschmidt-Clermont PJ. Deficient Smad7 expression: a putative molecular defect in scleroderma. PNAS 2002;99(6):3908-3913.
23. Pasini F, Brentani M, Kowalski L, Frederico M. Transforming Growth Factor β1, urokinase-type Plasminogen Activator and Plasminogen Activator Inhibitor-1 mRNA expression in head and neck squamous carcinoma and normal adjacent mucosa. Head Neck 2001;23:725-732.
24. Alessi MC, Bastelica D, Morange P, Berthet B, Leduc I, Verdier M, Geel O, Juhan-Vague I. Plasminogen Activator Inhibitor 1, Transforming Growth Factor-β1 and BMI are closely associated in human adipose tissue during mobid obesity. Diabetes 2000;49:1374-1380.
25. Dong C, Zhu S, Wang T, Yoon W, Goldschmidt-Clermont PJ. Upregulation of PAI-1 is mediated through TGF-β/Smad pathway in transplant arteriopathy. J Heart Lung Transplant 2002;21(9):999-1008.
26. Sheen-Chen S-M, Chen H-S, Sheen C-W, Eng H-L, Chen W-J. Serum levels of Transforming Growth Factor β1 in patients with breast cancer. Arch Surg 2001;136:937-940.
27. Saito H, Tsujitani S, Oka S, Kondo A, Ikeguchi M, Maeta M, Kaibara N. The expression of Transforming Growth Factor-β1 is significantly correlated with the expression of Vascular Endothelial Growth Factor and poor prognosis of patients with advanced gastric carcinoma. Cancer 1999;86:1455-1462.
28. Miyamoto H, Kubota Y, Shuin T, Torigoe S, Dobashi Y, Hosaka M. Expression of Transforming Growth Factor-beta 1 in human bladder cancer. Cancer 1995;75:2565-2570.
29. Lee C, Sintich S, Mathews E, Shah A, Kundu S, Perry K, Cho J-S, Ilio K, Cronauer M, Janulis L, Sensibar J. Transforming Growth Factor-β in benign and malignant prostate. Prostate 1999;39:285-290.
30. Wikstrom P, Stattin P, Franck-Lissbrant I, Damber JE, Bergh A. Transforming Growth Factor β1 is associated with angiogenesis, metastasis and poor clinical outcome in patients with prostate cancer. Prostate 1998;37:19-29.
31. Teicher BA. Malignant cells, directors of the malignant process : Role of transforming growth factor-beta. Cancer Met Rev 2001;20:133-143.
32. Allgayer H, Babic R, Grutzner K, Beyer B, Tarabichi A, Schildberg F, Heiss M. Tumor-associated proteases and inhibitors in gastric cancer: analysis of prognostic impact and individual risk protease patterns. Clin Exp Metastasis 1998;16:62-73.
33. Grondahl-Hansen J, Christensen I, Rosenquist Ch, Brunner N, Mouridsen H, Dano K, Blichert-Toft M. High levels of urokinase-type Plasminogen Activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 1993;53:2513-2521.
34. Ganesh S, Sier C, Griffioen G, Vloedgraven H, de Boer A, Welvaart K, van de Velde C, van Krieken J, Verheijen J, Lamers C, Verspaget H. Prognostic relevance of Plasminogen Activators and their inhibitors in colorectal cancer. Cancer Res 1994;54:4065-4071.
35. Itaya T, Suzuki K, Takagi I, Motai H, Baba S. Relationship between head and neck squamous cell carcinomas and fibrinolytic factors. Acta Otolaryngol (Stockh) 1996;suppl 525:113-119.
36. Kohler U, Hiller K, Martin R, Langanke D, Naumann G, Bilek K, Janicke F, Schmitt M. Tumor-associated proteolytic factors uPA and PAI-1 in endometrial carcinoma. Gynecol Oncol 1997;66:268-274.
37. Gleeson N, Gonsales R, Bonnar J. Plaminogen Activator Inhibitors in endometrial adenocarcinoma. Cancer 1993;72:1670-1672.
38. Horn L, Pippig S, Raptis G, Fischer U, Kohler U, Hentschel B, Martin R. Clinical relevance of urokinase-type plasminogen activator and its inhibitor type 1 (PAI-1) in squamous cell carcinoma of the uterine cervix. Aust N Z J Obstet Gynecol 2002;4:383-386.
39. Tjiong M, van der Vange N, ter Schegget J, Burger M, ten Kate F, Out T. Cytokines in cervicovaginal washing fluid from patients with cervical neoplasia. Cytokine 2001;14(6):357-360.
40. Chopra C, Dinh T, Hannigan E. Circulating serum levels of cytokines and angiogenic factors in patiens with cervical cancer. Cancer Invest 1998;16(3):152-159.
41. Wu H-S, Li Y, Chou C-I, Yuan C, Hung M, Tsai L. The concentration of serum Transforming Growth Factor beta-1 (TGF-β1) is decreased in cervical carcinoma patients. Cancer Invest 2002;20(1):55-59.

42. El-Sherif A, Seth R, Tighe P, Jenkins D. Decreased synthesis and expression of TGF-β1, β2 and β3 in epithelium of HPV-16-positive cervical precancer: a study by microdissection, quantitative RT-PCR and immunohistochemistry. J Pathol 2000;192:494-501.
43. Xu X-C, Mitchell M, Silva E, Jetten A, Lotan R. Decreased expression of retinoic acid receptors, transforming growth factor β, involucrin, and cornifin in cervical intraepithelial neoplasia. Clin Cancer Res 1999;5:1503-1508.
44. Farley J, Gray K, Nycum L, Prentice M, Birrer M, Jakowlew S. Endocervical cancer is associated with an increase in the ligands and receptors for transforming growth factor-β and a contrasting decrease in p27. Gynecol Oncol 2000;78(2):113-122.
45. Radhakrishna Pillai M, Jayaprakash P, Krishnan Nair M. Tumour-proliferative fraction and growth factor expression as markers of tumour response to radiotherapy in cancer of the uterine cervix. J Cancer Res Clin Oncol 1998;124:456-461.
46. Dickson J, Davidson S, Hunter R, West C. Pretreatment plasma TGFβ1 levels are prognostic for survival but not morbidity following radiation therapy of carcinoma of the cervix. Int J Radiation Oncology Biol Phys 2000;48(4): 991-995.
47. Kobayashi H, Fujishiro S, Terao T. Impact of urokinase-type plasminogen activator and its inhibitor type 1 on prognosis in cervical cancer of the uterus. Cancer Res 1994;54:6539-6548.
48. Moon H-S, Kim S, Ahn J, Woo B. Concentration of vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) in the serum of patients with cervical cancer: prediction of response. Int J Gynecol Cancer 2000;10:151-156.
49. Kersemaekers A, Fleuren GJ, Kenter G, Van den Broek L, Uljee S, Hermans J, Van de Vijver M. Oncogene alterations in carcinomas of the uterine cervix: overexpression of the epidermal growth factor receptor is associated with poor prognosis. Clin Cancer Res 1999;5(3): 577-586.
50. De Boer W, van Schadewijk A, Sont J, Sharma H, Stolk J, Hiemstra P, van Krieken J. Transforming Growth Factor β1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998;158:1951-1957.
51. De Boer W, Schuller A, Vermey M, van der Kwast, T. Expression of growth factors and receptors during specific phases in regenerating urothelium after acute injury in vivo. Am J Pathol 1994;145:1199-1207.
52. Ruiter D, Ferrier C, van Muijen G, Henzen-Logmans S, Kennedy S, Kramer M, Nielsen B, Schmitt M. Quality control of immunohistochemical evaluation of tumour-associated plasminogen activators and related components. European BIOMED-1 Concerted Action on Clinical Relevance of Proteases in Tumour Invasion and Metastasis. Eur J Cancer 1998;34:1334-1340.
53. Daneri-Navarro A, Macias-Lopez G, Oceguera-Villanueva A, Del Toro-Arreola S, Bravo-Cuellar A, Perez-Montfort R, Orbach-Arbouys S. Urokinase-type plasminogen activator and plasminogen activator inhibitors (PAI-1 and PAI-2) in extracts of invasive cervical carcinoma and precursor lesions. Eur J Cancer 1998;34 (4):566-569.
54. Riethdorf L, Riethdorf S, Petersen S, Bauer M, Herbst H, Jänicke F, Löning T. Urokinase gene expression indicates early invasive growth in squamous cell lesions of the uterine cervix. J Pathol 1999;189:245-250.
55. Nordengren J, Fredstorp Lidebring M, Gendahl P-O, Brünner N, Fernö M, Högberg T, Stephens R, Willen R, Casslen B. High tumor tissue concentration of plasminogen activator inhibitor 2 (PAI-2) is an independent marker for shorter progression-free survival in patients with early stage endometrial cancer. Int J Cancer 2002;97:379-385.
56. Bajou K, Noël A, Gerard R, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig N, Carmeliet P, Collen D, Foidart J. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med 1998;4(8):923-928.
57. Santin A, Hermonat P, Hiserodt J, Fruehauf J, Schranz V, Barclay D, Pecorelli S, Parham G. Differential Transforming Growth Factor-β secretion in adenocarcinoma and squamous cell carcinoma of the uterine cervix. Gynecol Oncol 1997;64:477-480.
58. Comerci J, Runowicz C, Flanders K, De Victoria K, Fields A, Kadish A, Goldberg G. Altered expression of Transforming Growth Factor -β1 in cervical neoplasia as an early biomarker in carcinogenesis of the uterine cervix. Cancer 1996;77:1107-1114.
59. Xu X-C, Mitchell M, Siva E, Jetten A, Lotan R. Decreased expression of retinoic acid receptors, Transforming Growth Factor β, Involucrin and Cornifin in cervical intraepithelial neoplasia. Clin Cancer Res 1999;5:1503-1508.

60. Kang S, Won K, Chung H-W, Jong H-S, Song Y-S, Kim S-J, Bang Y-J, Kim N. Genetic integrity of transforming growth factor β (TGF-β) receptors in cervical carcinoma cell lines: loss of growth sensitivity but conserved transcriptional response to TGF-β. Int J Cancer 1998;77:620-625.
61. Hasskarl J, Butz K, Whitaker N, Ullmann A, Dürst M, Hoppe-Seyler F. Differential cell cycle response of nontumorigenic and tumorigenic human papillomavirus-positive keratinocytes towards transforming growth factor-β_1. J Med Mol 2000;78:94-101.
62. De Geest K, Bergman C, Turyk M, Frank B, Wilbanks G. Differential response of cervical intraepithelial and cervical carcinoma cell lines to Transforming Growth Factor-β_1. Gynecol Oncol 1994;55:376-385.
63. Braun L, Dürst M, Mikumo R, Gruppuso P. Differential response of nontumorigenic and tumorigenic human papillomavirus type 16-positive epithelial cells to Transforming Growth Factor-β_1. Cancer Res 1990;50:7324-7332.
64. Chen T, de Vries E, Hollema H, Yegen H, Velluci V, Strickler H, Hildesheim A, Reiss M. Structural alterations of transforming growth factor-β receptor genes I human cervical carcinoma. Int J Cancer 1999;82:43-51.
65. Chu T-Y, Lai J-S, Shen C-Y, Liu H-S, Chao C-F. Frequent aberration of the transforming growth factor-β receptor II gene in cell lines but no apparent mutation in pre-invasive and invasive carcinomas of the uterine cervix. Int J Cancer 1999;80:506-510.
66. Lee S, Cho Y-S, Shim C, Kim J, Choi J, Oh S, Kim J, Zhang W, Lee J. Aberrant expression of SMAD4 results in resistance against the growth-inhibitory effect of transforming growth factor-β in the SiHa human cervical carcinoma cell line. Int J Cancer;2001;94:500-507.

Chapter 5