Cell-cell interactions in the gastrointestinal tumour-microenvironment
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Citation
Hawinkels, L. J. A. C. (2009, January 27). *Cell-cell interactions in the gastrointestinal tumour-microenvironment*. Retrieved from https://hdl.handle.net/1887/13432

Active TGF-β1 correlates with myofibroblasts and malignancy in the colorectal adenoma-carcinoma sequence

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Abstract

Transforming growth factor-β1 (TGF-β1), a cytokine involved in various stages of cancer, is produced as a latent complex and requires processing to become active. We have determined total and active TGF-β1 levels in homogenates of colorectal cancer tissue. Active TGF-β1 levels are increased in carcinomas but not in pre-malignant adenomas, in contrast to total TGF-β levels, which show a stepwise increase in the mucosa-adenoma-carcinoma sequence. Furthermore solely active TGF-β1 levels are associated with tumour stage and worse patients' prognosis. Active TGF-β1 levels correlated significantly with PAI-1, α-smooth muscle actin (SMA) and several matrix-remodelling proteinases. SMA levels are significantly increased in colorectal carcinomas compared to adjacent mucosa, but not in adenomas, indicating the absence of myofibroblast-accumulation in early tumour stages despite the enhanced total TGF-β1 levels.

This study shows that although total levels TGF-β are already enhanced in the pre-malignant colorectal adenomas, activation of TGF-β is indicative for malignant progression. In tumours, active TGF-β1 correlates significantly with SMA expression, indicating that tumour-promoting myofibroblasts might arise as a result of increased TGF-β1 activation. These data underline the significance of the interaction between malignant cells and (myo)-fibroblasts in the tumour-microenvironment, modulating the biologic behaviour of colorectal cancer.

Introduction

The tumour-microenvironment is increasingly recognized as an important factor in cancer progression[1-3]. Malignant cells induce host reactions, i.e. immune reactions, angiogenesis, and desmoplasia/fibrosis within and around tumour tissue. Tumour fibrosis consists of an accumulation of extracellular matrix-producing myofibroblasts and was initially regarded as the host's barrier against cancer dissemination[4]. Myofibroblasts are hyper-activated fibroblasts that simultaneous express vimentin, fibroblast-activation protein (FAP), and α-smooth muscle actin (SMA)[5]. Physiologically, myofibroblasts play a role in wound healing in which they rearrange the extracellular matrix (ECM) and contract wound edges by SMA mediated contractile properties[6]. In cancer, myofibroblasts circumvent apoptosis and remain hyper-activated, secreting high amounts of ECM molecules, cytokines, and matrix degrading enzymes including matrix metalloproteinases (MMPs)[7]. In addition, they influence the immune response[8], induce pro-invasive signals for tumour cells[9], and influence epithelial-mesenchymal transitions[7]. The origin of myofibroblasts has not been fully elucidated and is likely a combination of bone-marrow derived fibrocytes and transition of resident fibroblasts[10]. In cancer tissues the majority of myofibroblasts arise presumably from activation of fibroblasts by direct cell-cell contact, e.g. via EMMPRIN[5,11] or via growth factors like Transforming Growth Factor-β (TGF- β)[12,13].

TGF-β1 is synthesized as a latent, ECM-bound molecule which is activated via proteolytic and non-proteolytic pathways[14]. Activation of the latent TGF-β complex is crucial, because up-regulation without activation will have little biological and clinical consequences[15,16]. In cancer, TGF-β has tumour-inhibiting as well as tumour-promoting effects[17,18]. Although many details of the working mechanisms of TGF-β in cancer still have to be elucidated, the induction of SMA expression in fibroblasts, leading to myofibroblast trans-differentiation, is well established, at least *in vitro*[19].

In this study, we examined the presence of total and active TGF-β1 in the colorectal mucosa-adenoma-carcinoma sequence using tissue homogenates. The clinical relevance of TGF-β1 levels was evaluated by correlations with the grade and stage of the tumour and the survival of the patients. Furthermore, TGF-β1 levels were compared with various known target proteins like PAI-1, urokinase (uPA), and matrix metalloproteinases MMP-2 and MMP-9. To evaluate the role of TGF-β1 in myofibroblast generation, an ELISA for SMA, an established myofibroblast marker[19-21], was developed. The results of this study indicate the clinical

importance of especially active TGF-β1 levels and its relation with the quantity of myofibroblasts in colorectal carcinomas.

Materials and methods

Patient material and homogenates

Representative tissue specimens from endoscopically or surgically resected colorectal adenomas (Female n=14, Male n=21), carcinomas (Female n=77, Male n=102; Dukes A, n=3, B, n=105, C, n=45, D, n=26) and adjacent mucosa (n=179) were collected from the Departments of Oncologic Surgery and Gastroenterology of the Leiden University Medical Centre according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre. Macroscopic and microscopic parameters were obtained from the pathology reports, clinical data and follow-up were available for at least 10 years. The tissues were homogenised using a micro-potter in Tris/Tween-80 (pH 7.5) and protein concentrations were determined according to Lowry as previously described[22].

TGF-β1 ELISA

Total and active TGF-β1 levels were determined as described before[16]. In short, plates were coated O/N with TGF-β1 capture antibody, blocked, and 30 μl sample, with or without acid activation, was incubated to determine respectively endogenous active and total tissue TGF-β1 levels. Immune-detection was performed with biotinylated antibodies and a substrate reagent pack according to the manufacturer's protocol (R&D systems, Abington, UK).

ELISAs for uPA, tPA, PAI-1, PAI-2, MMP-2 and MMP-9

The levels of uPA, tPA, PAI-1, PAI-2, MMP-2 and MMP-9 were determined with established ELISAs as described before[22,23].

SMA ELISA

To determine SMA levels in colorectal tissue homogenates an ELISA was developed. Maxisorp 96-wells plates (Nunc, Denmark) were coated with rabbit polyclonal anti-SMA antibodies (Abcam, UK, 1:5000, stock 0.5 mg/ml) overnight at 4°C in coating buffer (NaHCO$_3$ buffer, pH 9.6). Plates were washed after each step with PBS containing 0.05% Tween-20 (PBST, Merck, Darmstadt, Germany). Non-specific binding was blocked with PBS containing 5% Tween-20, 0.05% NaN$_3$ during 1 hour at 37°C. Samples were 1:10 diluted in

PBS containing 1% Bovine Serum Albumin (BSA, Sigma, Darmstadt, Germany)/0.05% Tween-20 and allowed to bind 2 hours at 37°C. Immunodetection was performed with mouse monoclonal anti-SMA antibodies (stock 50 μg/ml, 1:32,000 diluted in PBS/1% BSA, clone ASM-1, Progen, Heidelberg, Germany) overnight at 4°C, biotinylated goat anti-mouse antibodies (Dako, Glostrup, Denmark, 1:4000, 2h RT) and Streptavidin-HRP (1:200, R&D systems). Colour development was performed with a substrate reagent kit according to the manufacturers protocol (Dy999, R&D systems). Absorbance was measured at 450 nm. SMA content was calculated in arbitrary units (AU)/mg protein, using a colonic muscle homogenate and TGF-β1 stimulated colonic fibroblasts as positive controls.

SMA ELISA validation

Colon cancer samples and corresponding mucosa samples were homogenised using the previously described conventional potter method (in Tris/Tween-80), or using an Ultra Turrax (IKA labortechnik, Staufen, Germany) on ice in 50 mM Tris/HCL buffer, pH 7.6 containing 1% Triton X-100 (BDH chemicals, Poole, UK) or using a mikro-dismembrator (Sartorius, Aubagne, France) during 30 sec at 3000 RPM followed by extraction with Tris-HCl, pH7.6 containing 1% triton X-100 for 5h at 4°C. After centrifugation, the extracts were used for TGF-β1 determination, whereas the pellets containing the insoluble actin filaments were further extracted with a 10 mM Tris/HCL buffer, pH 7.5 with 1% Sodium Dodecyl Sulphate (SDS), 1.25% β-mercaptoethanol and 2 mM EDTA by boiling for 3 minutes and subsequent sonification for 30 seconds[24]. Final protein concentrations were determined according to Lowry[25]. SMA levels were analysed using an ELISA and western blotting as described below. The sensitivity of the SMA ELISA was evaluated on four tissue samples using Turrax homogenation: Two colorectal cancer samples, one colon cancer derived liver metastasis and one gastrointestinal stromal tumour (GIST). For these samples ELISA-derived SMA levels were compared with western blot analysis and immunohistochemistry on the same samples. For Western blot analysis 10 μg protein was analysed on a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred to a nitrocellulose membrane (Whatman, Dasel, Germany) overnight. Non-specific binding was blocked with 0.2% gelatin (30 minutes at room temperature (RT)). After washing with PBS containing 0.05% Tween (PBST), immunodetection was performed with the antibody used as detection antibody in the ELISA, mouse monoclonal anti-SMA (1:5000, 2h RT). Next, blots were incubated with biotinylated goat-anti mouse antibodies, HRP-conjugated streptavidin (both Dako) and finally detection

was performed using a chemoluminescent substrate according to the manufacturer's protocol (Super Signal West Pico, Pierce, Rockford, IL, USA).

Immunohistochemistry

Tissue samples were fixated, dehydrated through graded alcohol and xylene and embedded in paraffin. Consecutive sections of four μm were deparaffinised and rehydrated. Subsequent antigen retrieval was performed by boiling in sodium citrate buffer pH 6.0 during 10 minutes. Immunostaining was performed using mouse monoclonal anti-SMA, mouse monoclonal anti-vimentin, mouse monoclonal anti-pan-cytokeratin (both Santa Cruz Biotechnologies, Santa Cruz, USA) and mouse monoclonal anti-desmin (clone 33, kindly provided by the Department of Pathology, Leiden University Medical Centre) as described previously [16]. In short, primary antibodies were incubated overnight at RT followed by detection with biotinylated goat anti mouse antibodies and StreptAvidin-Biotin Complex (All Dako). Staining was visualized using diaminobenzidine with H_2O_2. Positive- and negative controls (by omitting primary antibodies) were included for all stainings. Representative photomicrographs were taken using a Nikon Eclipse E800 microscope equipped with a Nikon DXM 1200 digital camera.

Isolation and characterisation of primary human colonic fibroblasts

Normal human mucosa was prepared from surgical obtained resection specimens by removing the muscle fraction. Tissue pieces of 5 mm were repeatedly treated with 0.5 M EDTA/PBS in a Wheaton chamber at 37°C until no epithelial cells were recovered from the tissue. The remaining tissue was washed with DMEM/F12 + Glutamax (Invitrogen, the Netherlands), containing 10% Fetal Calf Serum (FCS, Perbio Science, Erebodegem, Belgium), 10 mM HEPES, 50 μg/ml Gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen) and subsequently incubated in 75 cm^2 flasks until outgrowth of fibroblast-like cells was observed (7-10 days). The fibroblast origin of the cells was established by morphologic characteristics and immunohistochemical staining of cell-markers. Staining was performed by preparing cytospin preparations, fixation with ice cold acetone (10 min), followed by overnight incubation with primary antibodies against vimentin and pan-cytokeratin at 4°C and immunodetection. To generate myofibroblasts, subconfluent growing fibroblasts were stimulated with 5 ng/ml active recombinant human TGF-β1 (Peprotech, London, UK) during 24 hours. Myofibroblast differentiation was confirmed by positive staining for SMA (>95%) on cytospin preparations.

Statistical analysis

Statistical analyses were performed using the SPSS 12.0 Statistical Package (2004, SPSS Inc., Chicago, IL, USA). Group means are accompanied by standard errors of the mean. Differences between groups were calculated using the Student's t-test. Correlations between SMA and TGF-β levels were calculated according to Spearman. Log rank statistics were used for optimal cut-off point analysis. Survival curves were according to Kaplan and Meier. *P*-values ≤0.05 were considered significant.

Results

TGF-β1 levels in colorectal (pre-)malignancies

Total and endogenously active TGF-β1 levels determined in tissue homogenates of colorectal mucosa, adenomas and carcinomas are depicted in Figure 1. Total TGF-β1 levels were significantly increased in adenomas (116.8 ± 12.0 pg/mg protein, *P*<0.0005) and carcinomas (372.0 ± 21.4 pg/mg, *P*<0.0005) versus normal mucosa (41.2 ± 2.3 pg/mg) and adenomas (*P*<0.0005), but showed no clear relation with the presence of dysplasia in adenomas, nor with the differentiation grade (Figure 1A) or Dukes' stage of the carcinomas (Figure 1B).

In contrast, active TGF-β1 levels were not significantly increased in adenomas (9.2 ± 0.8 pg/mg, *P*=0.120) compared to colorectal mucosa (8.6 ± 0.8 pg/mg). In carcinomas there was a significant (32.5 ± 2.9 pg/mg, *P*<0.0005 versus normal mucosa) and stepwise increase with differentiation (Figure 1C) and Dukes stage of the tumour (Figure 1D).
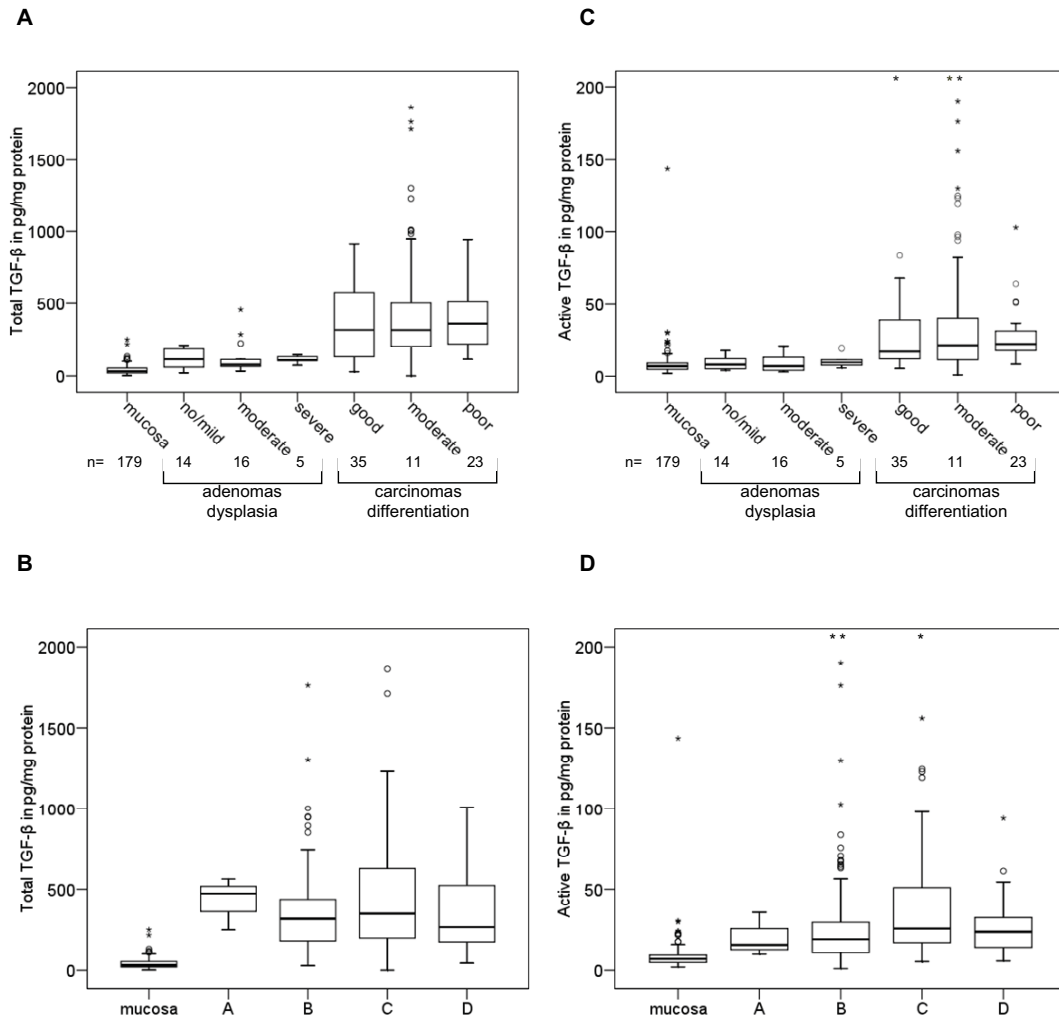
Figure 1. Total (A/B) and endogenous active (C/D) TGF-β1 levels in homogenates from respectively colorectal normal mucosa, adenomas and carcinomas. The adenomas are subdivided by dysplasia and the carcinomas by differentiation (A/C) and Dukes stage (B/D). The box-plots represent median, quartiles, extreme values (o) and outliers (∗) in pg/mg protein.

To establish the correlation between TGF-β1 levels and overall survival of the patients, we calculated optimal cut-off points. Only for active TGF-β1 a significant cut-off point was found (13.7 pg/mg, Figure 2A), resulting in a Log Rank of 4.06 with a *P*-value of 0.0439 (Figure 2B).
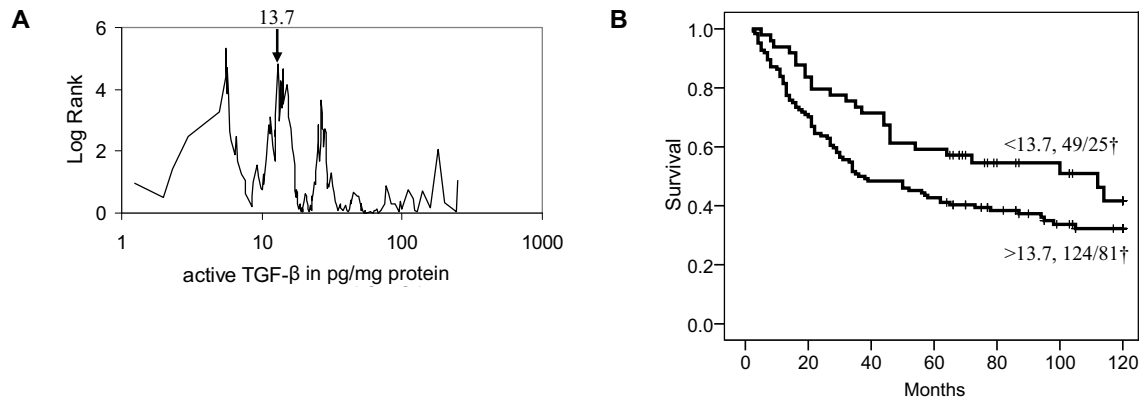
Figure 2. Optimal cut-point analysis for active TGF-β1 with respect to overall survival of colorectal cancer patients (A). Kaplan Meier survival curve showing that patients with high active TGF-β1 levels (>13.7 pg/mg protein) had significant worse survival than patients with low levels (Log rank 4.06, P=0.04).

Next, we analysed the correlation of TGF-β1 levels in colorectal tissues with various TGF-β1 target genes (Table 1). Active and total TGF-β1 correlated significantly with urokinase type plasminogen activator (uPA), the inhibitors PAI-1 and PAI-2 and matrix metalloproteinase MMP-2, but not with non-TGF-β1 depending tissue-type plasminogen activator (tPA). Interestingly, MMP-9 correlated with total but not with active TGF- β1.

Table 1. Correlation of TGF-β with target proteins in colorectal mucosal, adenomatous and carcinomatous tissue. Protein levels were determined using ELISAs in tissue homogenates.

	Active TGF-β (pg/mg)		Total TGF-β (pg/mg)	
Proteins (ng/mg)	Spearman's Rho	P	Spearman's Rho	P
uPA	0.491	<0.0005	0.650	<0.0005
tPA	0.164	0.057	-0.217	0.011
PAI-1	0.495	<0.0005	0.599	<0.0005
PAI-2	0.412	<0.0005	0.328	0.002
MMP-2	0.343	<0.0005	0.264	<0.0005
MMP-9	0.095	0.210	0.343	<0.0005

uPA: urokinase-type plasminogen activator, tPA: tissue-type plasminogen activator

PAI: plasminogen activator inhibitor, MMP: matrix metalloproteinase

Development and validation of a SMA-ELISA

Because TGF-β1 is *in vitro* able to differentiate fibroblasts into SMA-expressing myofibroblasts, we examined the relation between TGF-β1 and SMA levels in colorectal tissue samples using a newly developed ELISA. Our conventional potter/Tris-Tween based method of homogenization proved not stringent enough to extract detectable amounts of actin filaments from colorectal tissues (Figure 3A). Two alternative homogenization methods were capable of extracting detectable amounts of SMA from these tissue samples, as shown on the same western blot. Homogenization by Turrax or extracted pellets from dismembrator homogenates revealed similar levels of SMA. A Turrax muscle homogenate was subsequently used for evaluation of the ELISA. The absorbance units decreased linearly with increasing dilution of the samples (Figure 3B). As a more relevant positive control for our purposes we prepared a series of 5 homogenates consisting of cultures of respectively 0, 25, 50, 75 and 100% SMA positive myofibroblast (>95%) completed with inversely decreasing numbers of non-SMA expressing HT29 colon cancer cells. As expected, the ELISA levels showed a linearly increasing SMA signal with increasing myofibroblast levels (Figure 3C).
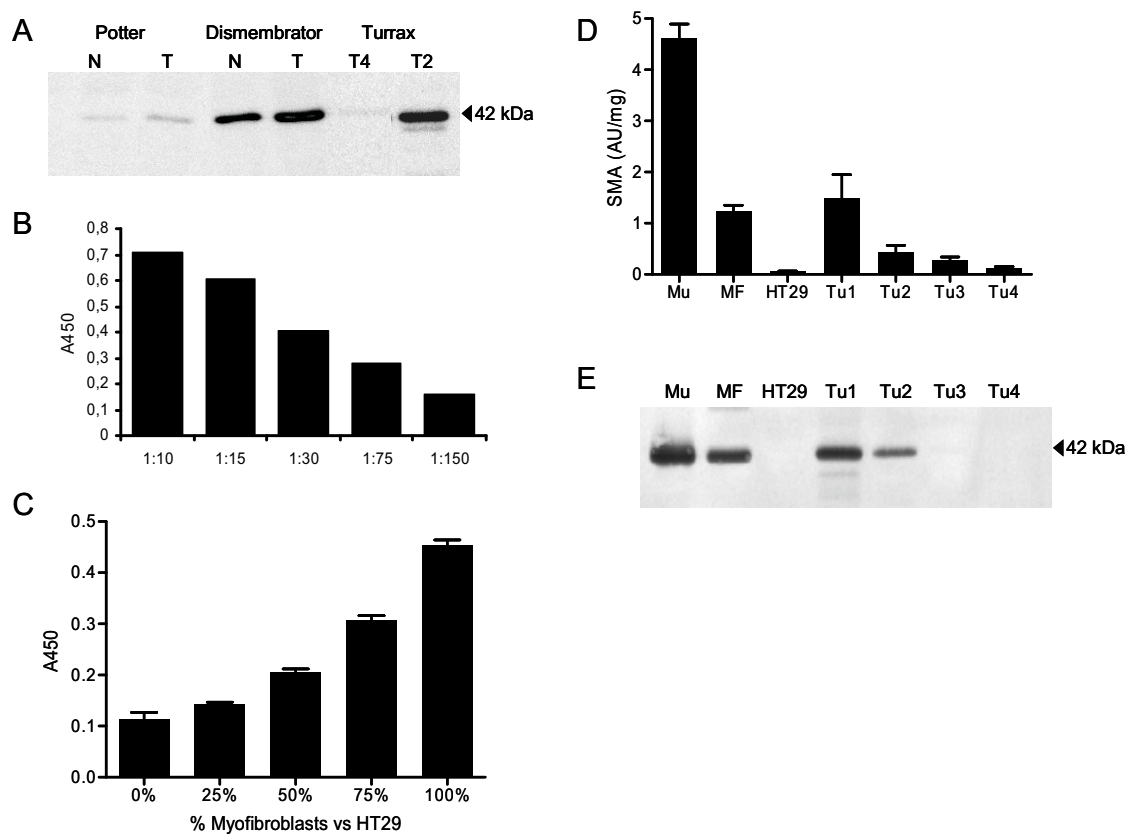

Figure 3. Legend see next page.

Figure 3. Development of an ELISA for smooth muscle actin (SMA). A) Western blot showing variations in SMA content of tissue homogenates generated with different extraction method. SMA absorption levels were measured in increasing dilution of a colonic muscle homogenate (B) or decreasing amount myofibroblasts versus non SMA expressing HT29 colon cancer cells (C). Four different gastrointestinal cancers show different SMA expression values (D) (Tu1 and Tu2 colon carcinoma, Tu3 colon cancer derived liver metastasis, Tu4 GIST located in the ileum). SMA western blot analysis (E) and immunohistochemistry (F) on the same samples to confirm the data obtained by ELISA.

One hundred percent HT29 homogenate gave no detectable signal above background. To evaluate the ELISA further, four different gastrointestinal tumours were selected showing varying levels in the ELISA (Figure 3D): Tu1 and Tu2 being colon carcinomas, Tu3 a colon cancer-derived liver metastasis and Tu4 a gastrointestinal stromal tumour (GIST, connective tissue carcinoma) located in the ileum. Figure 3e shows intense bands for the samples with the highest ELISA signal (Muscle, Tu1, Tu2) and low levels observed for Tu3 and 4.

The SMA-levels of all tissue samples were in between the absent signal of HT29 cells and the high signal of the myofibroblast homogenate. Immunohistochemistry confirmed that the SMA-signal as detected by western blot and ELISA was derived from myofibroblasts (Figure 3F). SMA immunoreactivity was mainly present in vimentin- and SMA-positive myofibroblasts. In normal colorectal tissues SMA-staining was observed in a thin layer of myofibroblasts along the crypt axis and in the muscularis mucosa beneath the colonic crypts (not shown). In carcinoma Tu1 very strong SMA expression was observed in the vast majority of the vimentin-positive myofibroblasts, whereas cytokeratin-positive tumour cells did not stain. Desmin staining, normally restricted to smooth muscle cells, revealed a few positive myofibroblasts. Carcinoma Tu2 also showed SMA expression in myofibroblasts, but to a lesser extent than observed in Tu1. Tu3, the colonic liver metastasis showed lower numbers of fibroblasts of which some were SMA positive and desmin negative. Finally the GIST (Tu4) showed almost exclusively vimentin-positive fibroblasts, but hardly any SMA positive cells, reflecting the data obtained by ELISA.
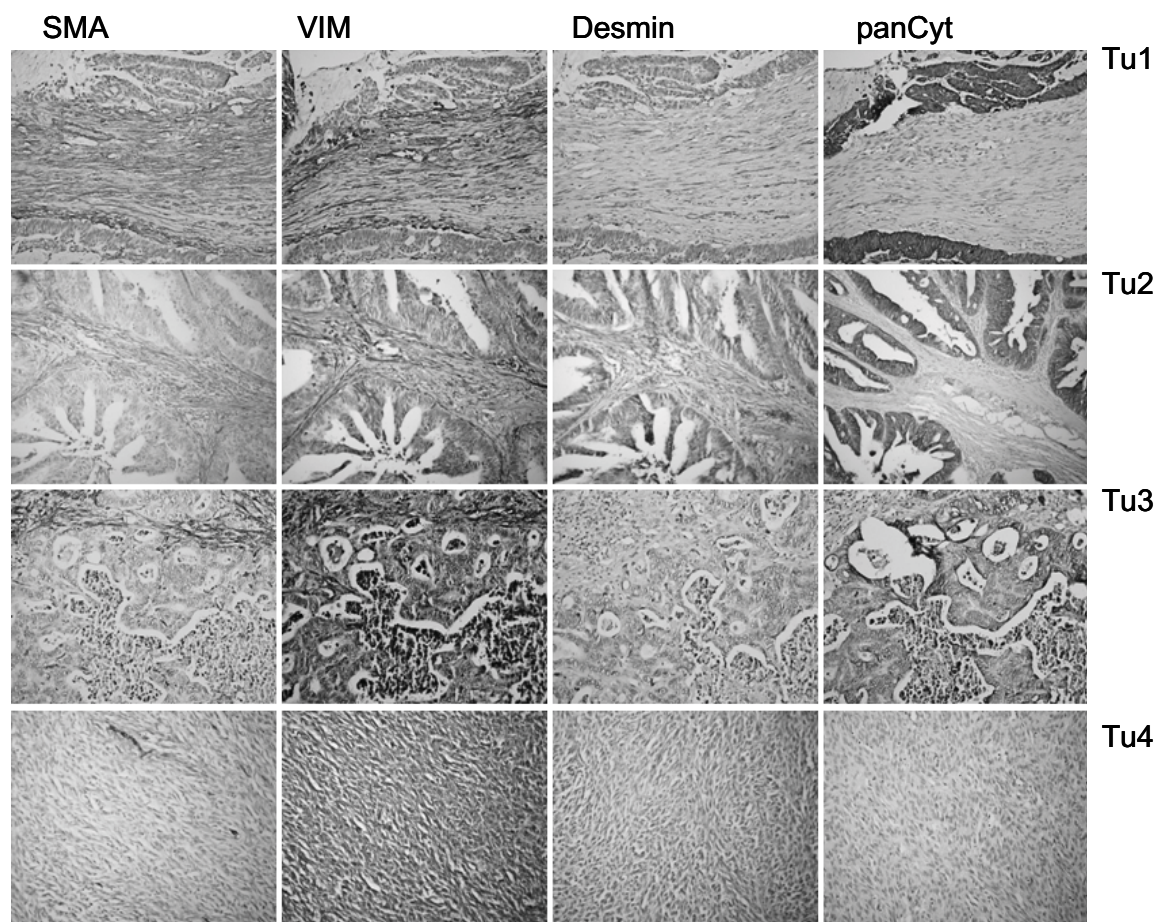
Figure 3F. Immunohistochemistry on Tu1-Tu4 samples, SMA = smooth muscle actin, myofibroblast and smooth muscle cell marker; Vim = vimentin, stromal cell marker; desmin, smooth muscle cell marker; pan-cytokeratin, epithelial marker). Full-colour illustration at page 204.

SMA levels in colorectal (pre-) malignancies

SMA levels were determined by ELISA in dismembrator type homogenates from normal colorectal mucosa, adenomas and carcinomas. Figure 4a shows significantly increased SMA levels for malignant tumours (1.50 ± 0.15 AU/mg protein, n=16) compared to normal mucosa (1.0 ± 0.08, n=18, $P=0.005$), whereas benign adenomas appeared to have even lower SMA expression levels (0.55 ± 0.07, n=17, P=0.0005) than mucosa. The SMA levels correlated significantly with active (Figure 4B, Rho= 0.558, P=0.0005) and total TGF-β1 (Figure 4C, Rho=0.599, P=0.0005) levels in these samples.
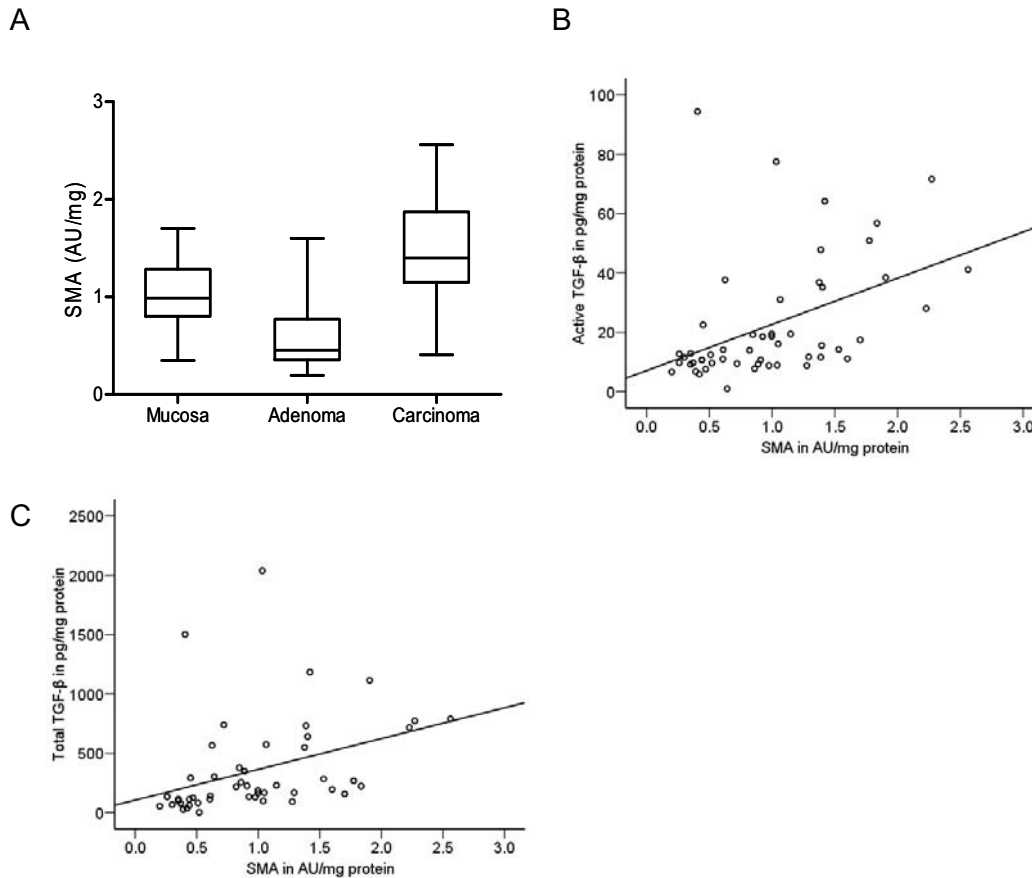
A

B

C

Figure 4. Smooth muscle actin (SMA) expression levels in colorectal (pre-)malignancies as determined by ELISA. A) The SMA concentration, representative for myofibroblast content is significantly increased in colorectal carcinomas (P=0.005), but not in adenomas. The box-plots represent median and quartiles. Correlation between SMA and active (B) and total (C) levels of TGF-β1 (both P=0.0005).

Discussion

The pluripotent growth factor TGF-β1 is produced as a latent precursor and therefore activation is a crucial regulating event. We have previously shown the clinical relevance of high levels of active TGF-β1 levels in gastric cancer[16], which we have now confirmed in a larger series of colorectal cancers and pre-malignant adenomatous polyps. Up-regulation of the latent TGF-β1 complex is already detectable early in the colorectal normal-adenoma-carcinoma sequence, whereas substantial increase of TGF-β1 activity occurs merely in carcinomas, showing an increasing stepwise relation with differentiation grade and Dukes stage. Furthermore, only high active TGF-β1 levels were related to worse survival of the cancer patients. Although several studies have been performed on the expression of TGF-β1 in colorectal carcinomas[26,27], up to now only one study also examined total and active TGF-β1

levels[28]. They found that higher total TGF-β1 protein expression was associated with increasing tumour stage, but did not find a significant difference in active TGF-β1 levels between normal and tumour samples. The discrepancy with our study could be due to sensitivity differences between the different assays used. Although on average their levels of total TGF-β1 were comparable with the results from our study, active TGF-β levels in normal tissue, requiring extremely sensitivity, are probably better detectable using a mild detergent-containing lysis buffer, as in our studies.

Myofibroblasts of normal colon, adenoma and carcinoma differ in the expression of 395 genes[6]. Although the origin of myofibroblasts is probably heterogeneous, TGF-β1 mediated trans-differentiation of resident fibroblasts seems to be a major source of myofibroblast aggregation in cancer[12]. Tumour-associated myofibroblasts promote the progression of carcinomas by modulation of invasion, angiogenesis and the immune-response[6, 7] and the recent association between the presence of tumour stromal cells with poor survival of colon carcinoma patients is in part due to myofibroblasts[29].

Various studies have assessed the presence and number of myofibroblasts in colorectal (pre-) malignancies, mainly based on immunohistochemical staining of SMA[20,30,31]. We have developed an ELISA capable of detecting SMA-levels in homogenates of colorectal tissues. We found that the method of tissue homogenization is crucial for measuring SMA levels. Conventional Triton X-100 extracts obtained according to the protocol recently described[32], showed low, hardly detectable SMA levels, because this technique is not stringent enough to extract the actin filaments[24]. However when resulting membrane fractions were boiled and treated with strong detergent and β-mercaptoethanol (to prevent clustering of the SMA filaments) this resulted in detection of levels of SMA, comparable to homogenation using a Turrax. Regardless of the two homogenization methods, a significant increase in SMA was observed in colorectal carcinomas compared to adjacent mucosa. Immunohistochemistry revealed the expression of SMA being present in myofibroblasts in different cancers. Desmin staining, normally restricted to smooth muscle cells, revealed also few desmin positive myofibroblasts, which has been described recently[33] and could indicate a minor additional source of myofibroblasts. SMA was also detectable in normal colorectal tissue probably because of the high expression of SMA in the muscularis mucosa. Interestingly, adenomas contained lower SMA levels than normal tissue, which could be explained by the fact that the majority of the cells is of epithelial origin and the relative absence of muscularis mucosa in these samples. In addition, SMA expression has also been shown to be dependent on the histological type of the adenoma[20]. The low levels of SMA in adenomas suggest that the up-

regulated levels of total TGF-β1 should be mainly of pre-malignant epithelial origin. The relative inactivity of this adenomatous TGF-β1 is probably due to the absence of an efficient activation mechanism and could result in suppression of malignant outgrowth of the tumour. Subsequent, over-activation of TGF-β1 in the adenomas will induce myofibroblast formation and in turn increased TGF-β1 expression which further validates tumour-associated myofibroblasts as therapeutic targets[34,35].

Besides induction of SMA in myofibroblasts TGF-β1 is also a regulator of extracellular matrix remodelling-associated genes. Colorectal tissue TGF-β1 levels correlated indeed significantly with the expression of urokinase and PAI-1 levels, two well-known TGF-β1 target genes, but also with PAI-2 and MMP-2. MMP-9 correlated with total but not with active TGF-β1, whereas the control proteinase tissue-type plasminogen activator did not correlate with TGF-β1 at all. PAI-1, urokinase and MMP-2 have been found in colorectal tumour-associated myofibroblasts as well as epithelial cells[31,36,37]. Recent studies have indicated that over-expression and efficient localized activation of both these proteolytic cascade systems are especially found at the interface between tumour cells and stromal cells[38-40]. Interestingly, plasminogen activation as well as MMP-activity have both been associated with TGF-β1 activation *in vitro*[41,42].

In conclusion, we have shown that total and active TGF-β1 levels are clearly related to the normal-adenoma-carcinoma sequence and active TGF-β1 is more indicative of malignant progression, tumour stage and survival of the patients than total TGF-β1. Furthermore, the correlation between active TGF-β1 levels and smooth muscle actin expression might indicate a prominent role for TGF-β1 in myofibroblast trans-differentiation.

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