

Ribozyme to TGF- β 1 mRNA abrogates immunosuppressive effects of human colorectal adenocarcinoma HRT-18 cells *in vitro* and *in vivo*

WOLFGANG DANIEL SCHMITT^{1*}, ANTJE SIEGERT^{2*}, SANDRA STEPHANIE LACH¹, STEPHAN FUHRMANN³, ANKE KONDLA⁴, PER SONNE HOLM⁵ and STEFFEN HAUPTMANN²

¹Institute of Pathology, Martin-Luther-University, 06097 Halle; ²Experimental Pharmacology and Oncology GmbH, 13122 Berlin; ³Department of Hematology, Oncology, and Tumor Immunology, HELIOS Klinikum Berlin-Buch, 13125 Berlin; ⁴Boehringer Ingelheim Pharma GmbH and Co. KG, 55216 Ingelheim am Rhein; ⁵Institute of Experimental Oncology and Therapy Research, Technical University, 81675 Munich, Germany

Received March 31, 2009; Accepted June 5, 2009

DOI: 10.3892/ijo_00000405

Abstract. Transforming growth factor- β 1 (TGF- β 1) is over-expressed in a variety of malignant epithelial tumors and was suggested to be a marker of colorectal cancer. Moreover, there is growing evidence that TGF- β 1 contributes to tumor progression by regulating tumor cell proliferation and differentiation, inducing a favorable tumor microenvironment, promoting migration and invasion, and suppressing macrophage cytotoxicity. Therefore, we stably transfected an anti-TGF- β 1 hammerhead ribozyme into the human colorectal adenocarcinoma cell line HRT-18. Expression of this ribozyme resulted in significant inhibition of TGF- β 1 expression on mRNA and protein level. This was associated with an enhanced tumor cell differentiation and a reduced tumor growth *in vivo*. The capability of tumor cells to suppress ROI production of co-cultivated human macrophages was abrogated in transfectants. Taken together, inhibition of TGF- β 1 in colorectal carcinoma cells might be an interesting therapeutic tool leading to reduced tumor cell growth and increased macrophage cytotoxicity. Thus, a gene-therapeutic approach using anti-TGF- β 1 ribozyme in combination with established anti-tumor agents is of great promise.

Introduction

TGF- β 1 is one of the most important profibrogenic growth factors of the human body. This mediator is overexpressed in

a variety of malignant tumors including ovarian (1), gastric (2), colorectal (3), liver (4), breast (5) and prostate carcinoma (6), malignant melanoma (7), glioblastoma (8) and pulmonary adenocarcinoma (9). Moreover, more than two thirds of the patients with colorectal carcinoma have elevated TGF- β 1 plasma levels in comparison to healthy volunteers. Plasma TGF- β 1 levels increase with higher Dukes' stage (10), and it has been shown that the TGF- β 1 level is of prognostic relevance in colorectal carcinomas, suggesting that this mediator plays a significant role in tumor progression (11,12).

In fact, TGF- β 1 modulates many aspects of tumor cell behavior ranging from tumor suppression at early stages to promotion of metastasis (13). Colon carcinoma cells can switch their response to TGF- β 1 from repression of proliferation as it is the case in normal epithelial cells to growth stimulation during carcinoma progression (14). This switch seems to be associated with mutations affecting at least one component of the TGF- β signaling pathway (15) or with an activated JNK-pathway (16). Additionally to this growth promoting role we have previously shown that TGF- β 1 is one of the key regulators of extracellular matrix composition (17) and therefore involved in formation of desmoplastic stroma which is typical for colorectal adenocarcinoma. TGF- β 1 also regulates integrin expression and acts as a motility factor (18). Thus, an increased expression and activation of TGF- β 1 results in the formation of favorable tumor cell micro-environment promoting tumor migration. Also, the TGF- β family is one of the most potent immunosuppressors described to date, since this mediator inhibits both cytotoxic lymphocytes and cytotoxicity of macrophages (19,20).

All these effects are mediated by a number of different pathways. Best known among them is the Smad-family, activated via TGF- β -receptors on the cell surface. But TGF- β can also activate the Erk, JNK and p38MAPK pathways (21). Activation of MAPK was shown in Smad-deficient cells (22) and even mutated TGF- β -receptor type I, defective in Smad-activation, mediated p38MAPK-activation by TGF- β 1 (23). This parallel activation of Smads and MAPKs is critical for proliferation, since Smads usually reduce, whereas JNK may

Correspondence to: Dr Steffen Hauptmann, Institute of Pathology, Magdeburger Str. 14, 06097 Halle, Germany
E-mail: steffen.hauptmann@medizin.uni-halle.de

*Contributed equally

Key words: TGF- β 1, hammerhead ribozyme, colorectal carcinoma, reactive oxygen species, macrophages

Table I. Primers used.

Oligos for	Sequences	Length of amplification product (bp)
Truncated TGF- β 1 target	5' TAATACGACTCACTATAGGACACGCAGTACAGCAAGGTC 3' 5' CGGGGCGGGGCGGGGCGGGGA 3'	199
Anti-TGF- β 1 ribozyme	5' TAATACGACTCACTATAGGTCATGTTGCT GATGAGTCCGTGAGGACGAAACAGCTGCTC 3'	
Ribozyme expression T7 BGH	5' TAATACGACTCACTATAGGG 3' 5' TAGAAGGCACAGTCGAGG 3'	316
Human TGF- β 1	5' GGCTGGAAGTGGATCCACGA 3' 5' GCAGGAGCGCACGATCATGTT 3'	243
Human aldolase	5' ATCCTGGCTGCAGATGAGTC 3' 5' GCCCTTGTCTACCTTGATGC 3'	248

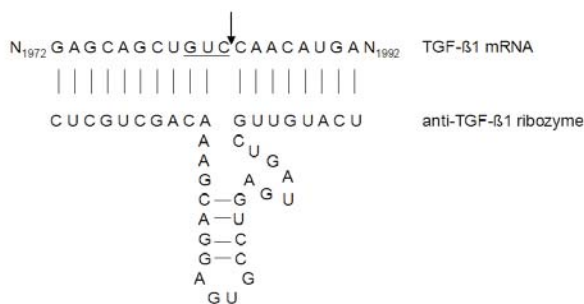


Figure 1. Sequence of the anti-TGF- β 1 hammerhead ribozyme, shown in the prototypical hammerhead conformation, indicating base-pairing interactions between the ribozyme and the TGF- β 1-encoding target mRNA. Arrow indicates the predicted site of cleavage.

promote proliferation. Finally, there are also hints for activation of phosphatidylinositol-3-kinase (PI3K), resulting in Akt-phosphorylation (24), and for an interaction with protein phosphatase 2A (PP2A) (25).

TGF- β 1 has a variety of effects and its inhibition may fundamentally alter tumor cell proliferation, differentiation, microenvironment, invasion and host immune response. Therefore, we constructed a ribozyme to inhibit TGF- β 1 expression. We used HRT-18 colorectal adenocarcinoma cells, which produce and respond to TGF- β 1, to investigate effects of diminished TGF- β 1 expression in respect to cell growth *in vitro*. Furthermore, we focused on cell adhesion and reactive oxygen intermediates (ROI) production of macrophages in co-culture with ribozyme-transfected HRT-18 cells. Finally, we investigated xenografts of native and ribozyme-transfected HRT-18 cells in nude mice.

Materials and methods

Cell culture. The human colorectal adenocarcinoma cell line HRT-18 was obtained from Cell Lines Service (Heidelberg,

Germany) and maintained in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (PAA, Cölbe, Germany). Monocyte isolation and cultivation as well as the co-culture model of MCTS (multicellular tumor spheroids) and macrophages were performed as described previously (20). The 25F9 macrophage phenotype used in these experiments represents the mature fraction of human macrophages.

Design and synthesis of anti-TGF- β 1 hammerhead ribozyme
Ribozyme-mediated RNA cleavage in vitro. To design an anti-TGF- β 1 ribozyme, a GTC site in exon 7 at nucleotide 1983 within the coding sequence for the mature peptide of the human TGF- β 1 cDNA sequence (accession no. X02812) was selected and tested according to Gibson and Shillitoe (26). The ribozyme and its respective target sequence are shown in Fig. 1.

Cleavage activity of this ribozyme was tested in a cell-free *in vitro* assay as described by Wichert *et al* (27). Oligo sequence for the anti-TGF- β 1 ribozyme and primer sequences for amplification of truncated TGF- β 1 target transcript out of total cDNA of TGF- β 1 expressing HRT-18 cells are shown in Table I.

Plasmid construction and stable transfection of HRT-18 cells. The ribozyme sequence was cloned into the eukaryotic expression vector pcDNA3.1/V5/HIS-TOPO (Invitrogen, Carlsbad, USA). Correct sequence and orientation of the insert were confirmed by sequencing. Circular pcDNA3.1 vector without ribozyme served as a negative control (mock). Transfections were performed using TransFast™ according to the manufacturer's instructions (Promega, Mannheim, Germany). Several stable cell clones resistant to G418 sulfate (300 μ g/ml) were selected after 3 weeks and expanded for screening of ribozyme- and TGF- β 1 expression. Each of the clones was separately analyzed and one representative mock and ribozyme clone chosen for detailed characterization in comparison to non-transfected HRT-18 cells.

Reverse transcription-polymerase chain reaction (RT-PCR); real-time-PCR. Total RNA was isolated by the phenol/isothiocyanate method using TRIzol-reagent (Gibco, Eggenstein, Germany). To compensate for variation in reverse transcription efficiency, cDNA from three independent reactions was pooled. Relative quantification of the TGF- β 1 mRNA was performed by real-time PCR using the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany) according to the manufacturer's instructions in the presence of 3 mM MgCl₂ and the primers shown in Table I on a LightCycler® instrument (Roche). The housekeeping gene aldolase served as an external standard. The annealing temperatures were 55 and 58°C for TGF- β 1 and aldolase, respectively. Standards for TGF- β 1 and aldolase were generated by cloning their PCR products in the pCR2.1 vector (Invitrogen). Plasmids were cultured in *E. coli* using standard procedures. The Maxi-Prep kit (Qiagen, Hilden, Germany) was used to isolate and purify the vector. A quantification curve was calculated facilitating serial dilutions of the standard vectors (10⁸-10³ copies) when amplified with the unknown samples within the same LightCycler run. Data calculations were performed by the second derivative maximum method. TGF- β 1 and aldolase copies were measured in three different runs and a ratio of TGF- β 1/aldolase copies was calculated. Only runs with an error <0.05 and an amplification efficiency \geq 1.8 were accepted. The mean ratio and standard deviation of three runs is shown.

TGF- β 1 enzyme-linked immunosorbent assay (ELISA). Supernatants of tumor cells were collected after 24 h of culture in serum-free medium, centrifuged and stored at -80°C. TGF- β 1 concentrations were measured using the human Quantikine™ TGF- β 1 immunoassay (R&D Systems, Bad Nauheim, Germany).

Proliferation assay. Proliferation was assessed by counting the cells with a CASY cell counter (Schärfe Systems, Reutlingen, Germany). Cells were seeded in 6-well cluster plates and two wells of each clone were counted at days 2, 4, 6 and 9.

Adhesion assay. Microwell cluster plates (96) were coated with Matrigel® Basement Membrane Matrix (Becton-Dickinson, Heidelberg, Germany) diluted in serum-free RPMI-1640 medium (1 mg/ml; 55 μ l/well). Non-specific binding was blocked by incubating with 1% bovine serum albumin/PBS. Seven wells per group were plated with cells at a density of 3.5x10⁴ cells/100 μ l/well and incubated for 90 min at 37°C with 5% CO₂ in a humidified incubator. Medium was aspirated, attached cells were fixed in 70% ethanol at -20°C for 10 min and stained with crystal violet. After plates were rinsed with water, 0.2 Triton X-100/0.1 N HCl was added to solubilize the cells and the absorbance of the solution was measured at 590 nm.

Chemiluminescence assay. Production of ROI was measured on a Luminoskan RS luminometer (Labsystems, Helsinki, Finland) as described previously (20). Briefly, macrophages were cultured in 96-well plates in the presence of tumor cells

(ratio 10:1) for 24 h. ROI production was stimulated with 0.1 μ M PMA 15 min after adding 100 μ M Lucigenin to each well.

Xenografts in SCID-mice. Female BALB/c nude mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed in an animal facility under specific pathogen-free conditions. All animal facilities and experiments were approved by local authorities. At the age of 6 weeks, 2.5x10⁶ HRT-18 cells (native, ribozyme-transfected and mock clone, respectively) in 1xPBS solution were injected subcutaneously. In macrophage co-culture experiments, 2.5x10⁵ cells of the 25F9 macrophage phenotype were added. Xenograft growth was measured twice a week and tumor volumes were calculated. On day 30, mice were sacrificed and tumors isolated. One half of each tumor was frozen in liquid nitrogen immediately, whereas the other half was fixed in 4% neutral-buffered formalin solution for immunohistochemical studies. TGF- β 1 mRNA expression was determined in a 5 mm cube of frozen tissue using real-time PCR as described previously.

Histological and immunohistochemical investigation of tumor tissue. Formalin-fixed tissue was processed using a routine wax-embedding procedure for histological examination, standard H&E slides were stained, and two pathologists independently analyzed tumor differentiation and the degree of necrosis.

For immunohistochemical staining cell pellets of native, ribozyme-transfected and mock clone HRT-18 cells were embedded in wax using standard procedures. Slides (2 μ m) were deparaffinized using xylene and a series of alcohol-water solutions. Antigens were retrieved by incubation in citrate buffer (pH 6.0) for 5 min in a steam cooker. Staining was performed using Dako ChemMate detection kit K5005 (Dako, Hamburg, Germany) following the instruction manual. The following antibodies were used: β -catenin (BD Transduction, Heidelberg, Germany, 1:5000), CEA (Novocastra/Leica Microsystems, Wetzlar, Germany, 1:100), E-cadherin (Dako, 1:50), MUC2 (Novocastra, 1:100), MUC4 (Zymed/Invitrogen, Karlsruhe, Germany, 1:100), MUC5AC (Novocastra, 1:50) and MUC6 (Novocastra, 1:50).

After haematoxylin counterstaining, slides were analyzed by two pathologists and the percentage of staining was determined.

Statistics. All data shown were obtained from at least three independent experiments and were expressed as mean \pm SD. Statistical significance of *in vitro* experiments was determined by Student's t-test, for xenograft growth by ANOVA trend test, and for TGF- β 1 expression as well as vital tumor masses in xenografts by Mann-Whitney U test. P-values <0.05 were considered to show a statistically significant difference.

Results

Ribozyme-mediated cleavage of truncated TGF- β 1 mRNA in a cell-free assay. The ability of the anti-TGF- β 1 ribozyme to cleave an *in vitro* transcribed truncated fragment of the TGF-

Table II. TGF- β 1 expression in HRT-18 and transfected cells.

	HRT-18	Mock	Ribozyme
TGF- β 1 mRNA			
Ratio of relative mRNA copy numbers	3.05 \pm 0.29	8.72 \pm 0.6	1.07 \pm 0.22
TGF- β 1/aldolase ^a			
TGF- β 1 protein			
TGF- β 1/24 h/10 ⁷ cells ^b	39 pg/ml	78 pg/ml	8 pg/ml

^aMeasured by real-time PCR; ^bmeasured by human Quantikine TGF- β 1 immunoassay.

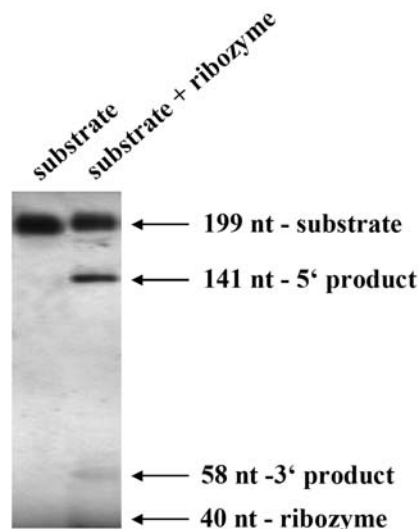


Figure 2. *In vitro* cleavage of a truncated TGF- β 1 RNA substrate by the anti-TGF- β 1 hammerhead ribozyme. Substrate (150 nM) was incubated with 150 nM ribozyme at 37°C for 90 min in the presence of 12 mM MgCl₂. Samples were size-fractionated on an 8% polyacrylamide gel containing 7 M urea. Cleavage products in the predicted size of 141 and 58 nt, and 199 nt substrate were identified by autoradiography.

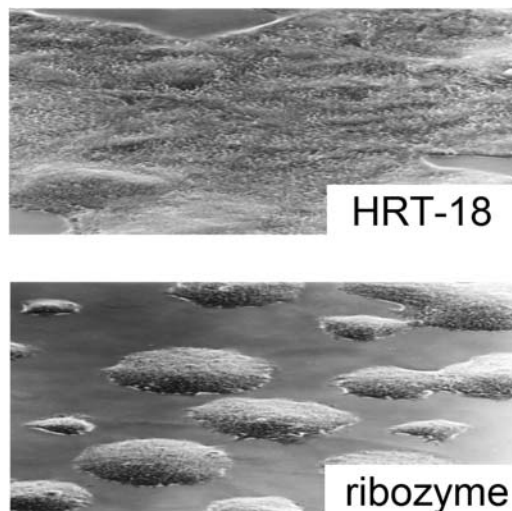


Figure 3. Growth pattern of native HRT-18 cells compared to ribozyme-transfected cells. Macroscopic photos show a confluent layer of the native cell line, whereas ribozyme-transfected cells spread in clusters.

β 1-encoding mRNA was shown in a cell-free system. The ribozyme degraded ~40% of the truncated TGF- β 1-encoding mRNA fragment after 1.5 h at physiological pH and temperature, with equimolar concentrations of ribozyme and TGF- β 1 mRNA (Fig. 2).

Expression of anti-TGF- β 1 ribozyme and decreased expression of TGF- β 1 in HRT-18 cells. Clones expressing anti-TGF- β 1 ribozyme (30) and 6 mock-transfected clones of the human colorectal adenocarcinoma cell line HRT-18 were isolated and TGF- β 1 expression was investigated. One representative clone of each group was selected for further detailed analysis.

Relative quantification of TGF- β 1 mRNA in relation to the house-keeping gene aldolase by real-time PCR showed diminished TGF- β 1 mRNA levels in the ribozyme clone, but increased TGF- β 1 mRNA level in the mock clone compared to HRT-18 cells without transfection. In parallel, secreted TGF- β 1 protein was reduced ~80% in the ribozyme clone measured by ELISA (Table II).

Inhibition of TGF- β 1 results in a different growth pattern, minor changes in protein expression, reduced cell adhesion on Matrigel, but has no effect on proliferation in vitro. While native HRT-18 and mock cells grow as confluent monolayer, the ribozyme-transfected cell clone forms slightly exalted clusters (Fig. 3).

Expression of a number of colorectal protein markers was slightly changed, ribozyme-transfected cells showed appearance of CEA (carcinoembryonic antigen), reduction of MUC5AC protein (mucin 5, subtypes A and C) and loss of MUC6 (mucin 6). All other proteins investigated showed no changes (Table III).

Adhesion was measured on Matrigel, a reconstituted basement membrane. HRT-18 cells adhered poorly to plastic surfaces of the culture well. The adhesion of the diminished TGF- β 1-expressing ribozyme clone on Matrigel was significantly reduced by ~36%, whereas the adhesion of the mock clone with higher TGF- β 1 expression increased ~35% (Fig. 4).

Proliferation investigated by cell counting over a period of 9 days showed no significant varieties of proliferation between untransfected HRT-18 cells, ribozyme- and mock-transfected clone in the presence of 10% fetal calf serum (data not shown).

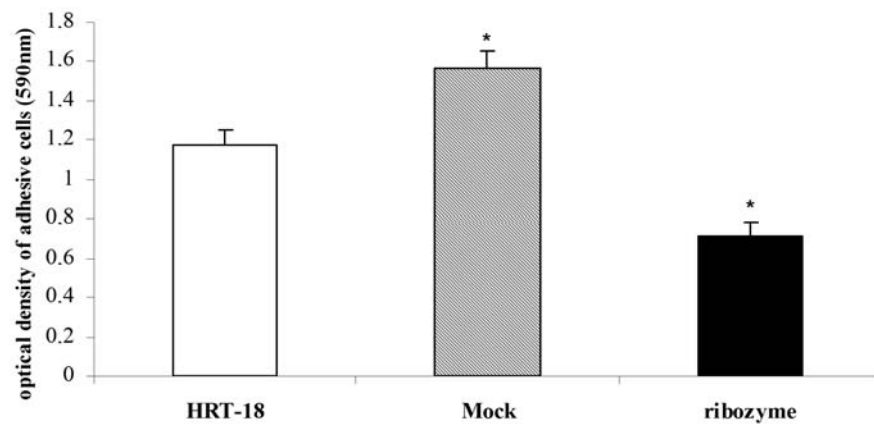


Figure 4. Cell adhesion of either HRT-18 cells, mock cells, HRT-18 clone transfected with vector, and ribozyme cells, HRT-18 clone transfected with the anti-TGF- β 1-ribozyme vector on Matrigel measured by crystal violet staining 90 min post-seeding. Adhesion of ribozyme-transfected cells was significantly decreased, whereas adhesion of mock-transfected cells was significantly increased compared to untransfected HRT-18 cells. Data of adhesive cells, mean \pm SD of three independent experiments are shown. *Significant ($p < 0.05$) in comparison to HRT-18 untransfected cells.

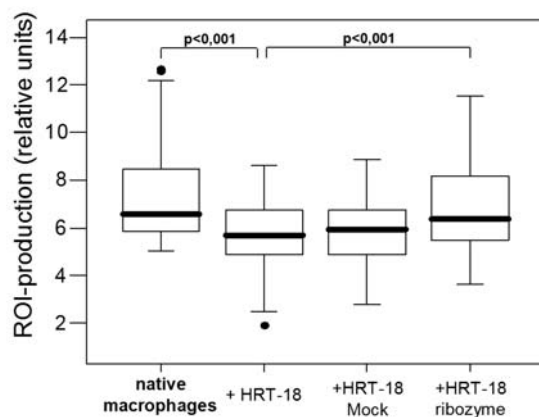


Figure 5. Production of ROI in tumor cell - macrophage co-cultures. Mature macrophage phenotype 25F9 was co-cultured with multicellular tumor spheroids of either colorectal adenocarcinoma cells HRT-18, mock cells - HRT-18 clone transfected with vector, and ribozyme cells - HRT-18 clone transfected with the anti-TGF- β 1-ribozyme vector. On day 4 of spheroid culture, macrophages were added for 24 h. ROI were measured after stimulation with PMA by the lucigenin-amplified chemiluminescence method. ROI production of macrophages was significantly inhibited by HRT-18 cells, but inhibition was abolished by expression of the anti-TGF- β 1 ribozyme in the ribozyme-transfected clone.

Table III. Immunoreactive profile of native and ribozyme-transfected HRT-18 cells.

	HRT-18 native	HRT-18 ribozyme
β -catenin	+	+
E-cadherin	+	+
CEA	-	partly +
MUC2	-	-
MUC4	-	-
MUC5AC	+	weakly +
MUC6	+	-

Effect of the ribozyme expression in HRT-18 MCTS on the production of ROI in co-culture with the 25F9 macrophage phenotype. Previously, we showed the inhibition of ROI production of different macrophage phenotypes by HRT-18 cells as well as an inhibition of ROI by recombinant TGF- β 1 (20). Now, we measured ROI production of the mature macrophage phenotype 25F9 in co-culture with MCTS of the ribozyme-transfected HRT-18 clone and found that decreased expression of TGF- β 1 in HRT-18 cells causes abolishment of the inhibition of ROI production of 25F9 macrophage phenotype as observed with untransfected HRT-18 cell spheroids and the mock clone (Fig. 5).

Xenografts of anti-TGF- β 1 ribozyme transfected tumor cells show reduced TGF- β 1 expression, but slightly enhanced tumor growth. Xenografts of either native HRT-18 or ribozyme-transfected HRT-18 cells were generated with and without the addition of human macrophages, respectively. Xenografts of ribozyme-transfected cells showed in both cases a slightly increased tumor growth. Due to the limited number of animals this increase did not reach statistical significance ($n=5$, $p=0.08$, respectively). The addition of macrophages retarded tumor growth in native and ribozyme-transfected xenografts whereas the ribozyme-transfected ones were statistically significant ($p=0.03$) despite the comparatively low number of cases (Fig. 6).

TGF- β 1 levels in the xenograft tumors were quantified to assess the sustainability of the ribozyme induced TGF- β 1-suppression. As expected, xenografts consisting of ribozyme-transfected cells contained reduced TGF- β 1 levels compared to those of native HRT-18 cells. Due to the limited number of samples, this difference did not reach statistical significance ($p=0.06$). Xenografts of native or ribozyme-transfected cells together with macrophages showed TGF- β 1 expression levels between those extremes, whereas the contribution rate of the two cellular fractions was not determined (Fig. 7).

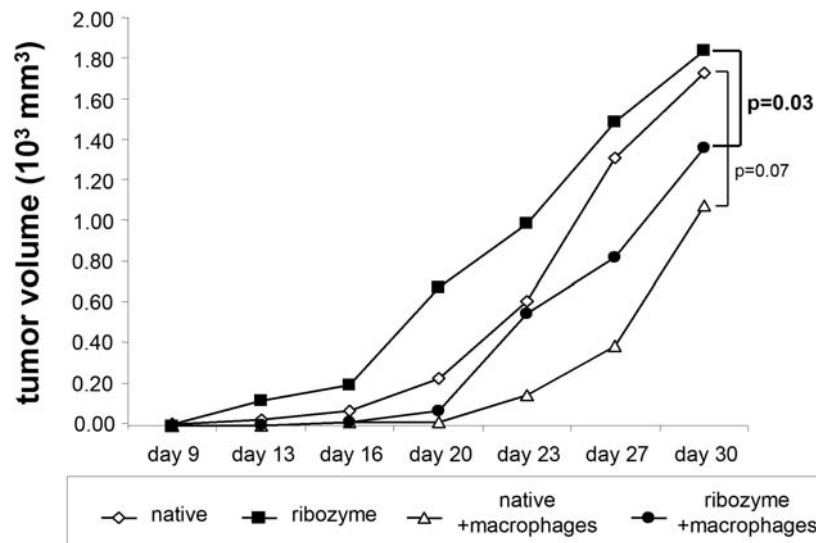


Figure 6. Growth of xenografts in nude mice. Native as well as ribozyme-transfected HRT-18 cells were injected in nude mice alone or together with human macrophages. Tumor volumes are calculated based on tumor diameters. In general, tumor sizes of ribozyme-transfected cells increased faster than those of native cells (not significant, $p=0.08$, respectively). The addition of macrophages reduced tumor growth in native ($p=0.07$) as well as ribozyme-transfected cells ($p=0.03$).

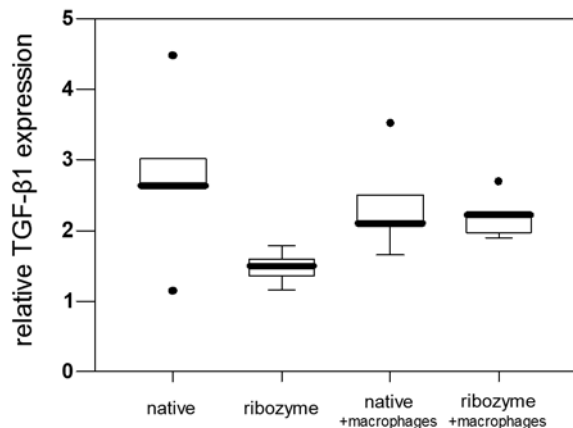


Figure 7. Relative TGF- β 1 expression in xenografts determined in a 5 mm cube of frozen tissue by real-time PCR. Ribozyme-transfected HRT-18 cells produced lower amounts of TGF- β 1 ($p=0.06$). Addition of macrophages reduced TGF- β 1 production of native HRT-18 cells slightly, but led to an increase in tumors of ribozyme-transfected cells ($p=0.09$).

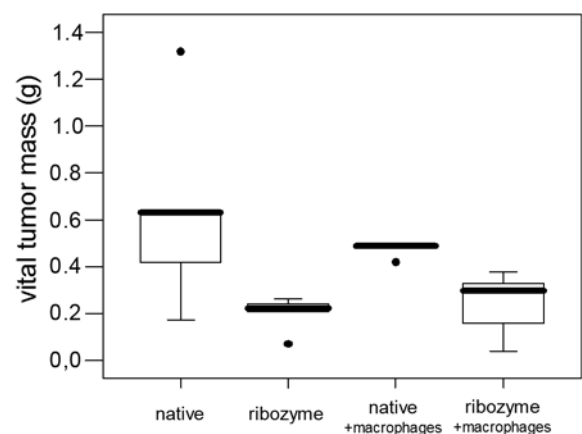


Figure 8. Final vital tumor masses of xenografts. Total masses of tumor tissue were measured after the sacrifice of mice. Vital tumor masses were calculated after histological determination of percentages of necrosis. Ribozyme-transfected HRT-18 cells showed lower vital tumor masses compared to native HRT-18 cells ($p=0.09$).

Vital tumor masses of xenografts of anti-TGF- β 1 ribozyme-transfected tumor cells were lighter than that of native HRT-18 cells. To account for the different degrees of necrosis observed in the xenografts, vital tumor masses were calculated based on total masses and percentages of necrosis. Weight of ribozyme-transfected xenografts was reduced to one third compared to tumors of native HRT-18 ($n=5$, $p=0.09$). This effect was slightly reduced when macrophages were added (Fig. 8).

Discussion

TGF- β 1 is produced by a number of malignancies and has been suggested to be a prognostic marker as well as a therapeutic target in patients with colorectal carcinoma (28).

We constructed an anti-TGF- β 1 ribozyme and investigated its effects in stably transfected HRT-18 colorectal adenocarcinoma cells, which spontaneously produce this mediator. Additionally, we generated xenografts of these cells in nude mice, partly with addition of human macrophages.

Hammerhead ribozymes diminish gene expression by cleaving numerous copies of its respective mRNA-substrate (26). The herein described anti-TGF- β 1 hammerhead ribozyme is directed against the human TGF- β 1 mRNA and does not cleave human TGF- β 2 or - β 3 mRNA (data not shown). We were able to show that this ribozyme has endoribonucleolytic activity in a cell-free system. Stable transfection resulted in a significant inhibition of TGF- β 1 expression on mRNA and protein level. Remarkably, TGF- β 1 expression was increased in all mock clones (which were transfected with the empty

pcDNA3.1 vector) compared to untransfected HRT-18 control cells. We also found this vector effect in other cell lines (A27/80, OVCAR-3, 257P and 181P), which is in accordance with results from other authors (29).

Although secretion of TGF- β 1 was reduced by 80% in the ribozyme clone, this inhibition did not change proliferation of HRT-18 cells *in vitro*. In contrast, other studies described a growth-inhibiting effect of TGF- β 1 in human colon carcinoma FET cells, mediated by TGF- β 1 antisense treatment (30). Similar, negative growth regulation by TGF- β 1 was reported in a murine high-grade glioma model, in which TGF- β inhibited tumor growth and enhanced apoptosis (31). These effects are not surprising as TGF- β 1 physiologically inhibits proliferation in normal epithelial cells. However, in transformed cells TGF- β 1 usually has no anti-proliferative effect or might act as growth stimulator (32). Effects of TGF- β 1 on proliferation are cell line-dependent and may even differ in various experimental settings. The determination whether TGF- β 1 has a positive or negative effect on proliferation is dependent on the current status of the TGF- β 1 signaling system as a whole. This comprises the amount of active TGF- β 1, expression of functional receptors and integrity of downstream signaling pathways (21,33). The resulting effect of TGF- β 1 seems to change during tumor progression in colon carcinoma (14) and was also investigated in a model of breast cancer progression (34). In the latter case, a switch was described within a single cell line at different stages of carcinogenesis. Furthermore, TGF- β has tumor-suppressive effects on normal epithelium and low-grade invasive cancer, but acts pro-metastatically in high-grade invasive cancer.

It has also been discussed, that TGF- β 1 is an important factor in regulation of cell adhesion molecules and extracellular matrix production. It is thought to play a role in the composition of the tumor stroma and for modulation of tumor cell migration. In TGF- β 1 antisense-transfected colon carcinoma FET cells α 5-integrin was reduced which led to reduced adhesion of tumor cells to fibronectin (35). In our model, diminished TGF- β 1 expression in HRT-18 ribozyme clone significantly decreased adhesion of tumor cells to Matrigel basement membrane matrix. Similar results are described in hepatocellular carcinoma cells in which TGF- β 1 stimulated α 3-integrin expression and triggered invasiveness (36).

In addition to these effects, it is known that TGF- β 1 acts as a very potent immunosuppressor. Together with other factors secreted by tumor cells, e.g. interleukin-10 or prostaglandin E2, it suppresses anti-tumor activity of immunocompetent cells which are no longer able to reject the tumor (19,37). Especially, tumor-associated macrophages are converted to an anti-inflammatory behavior and promote tumor cells (38). A recent review summarized the immunosuppressive effects of TGF- β 1 on dendritic and effector T cells (39), whereas it had been shown that toxicity of cytotoxic T cells can be restored by TGF- β depletion (40). On the basis of these observations, inhibition of TGF- β 1 should attract inflammatory macrophages, restore cytotoxicity of T cells and hopefully switch the phenotype of tumor-associated macrophages back to an anti-tumor behavior. Support for this hypothesis comes from a study on the non-immunogenic rat prostate cancer line MATLyLu. After downregulation of TGF- β 1 by antisense strategy tumor

incidence in normal rats was significantly decreased, whereas tumor incidence in immunodeficient rats was 100% (41).

Anti-tumoral cytotoxicity of macrophages is partly exerted by ROI (42), which can have anti-tumoral as well as tumor-promoting effects (43). We have previously shown that HRT-18 cells suppress the production of ROI by human macrophages in an *in vivo*-like co-culture model (20). Now we could demonstrate that this suppression is mediated in part by tumor-produced TGF- β 1. In our xenograft model, ribozyme-transfected tumors showed generally reduced vital tumor masses. This reduction was also seen in ribozyme-transfected tumor cells in combination with human macrophages, although the experimental setting did not allow a clear differentiation between direct ribozyme-effects and overlapping effects of restored ROI production of tumor-associated macrophages. Nevertheless, inhibition of TGF- β 1 expression in tumor cells might maintain cytotoxic activities of tumor-associated macrophages *in vivo*.

Taken together, several effects of TGF- β 1 influence the behavior of tumors *in vivo* and result in different net effects. In our study, the constructed anti-TGF- β 1 ribozyme reduces cell adhesion and increases tumor necrosis in xenografts, with no significant changes in proliferation of the cell line HRT-18 used in this work. This positive correlation between TGF- β 1 and tumor progression is in accordance with other studies, i.e. in hepatocellular carcinoma (36), pulmonary adenocarcinoma (9), prostate (6) and colon cancer (3,10,11,32,44).

Apart from immediate effects on tumor cells, inhibition of TGF- β 1 would also diminish immunosuppressive effects on macrophages and enhance their cytotoxic ROI production in the microenvironment of tumors. This reconstitution of the locally suppressed immune system might be an important factor for tumor elimination in addition to established tumor agents.

Acknowledgements

This work was supported by Deutsche Krebshilfe grant IO-I523 and a generous donation of the family and friends of Mr. Jürgen Lühr, Wrestedt, Germany.

References

1. Higashi T, Sasagawa T, Inoue M, Oka R, Shuangying L and Saijoh K: Overexpression of latent transforming growth factor-beta 1 (TGF-beta 1) binding protein 1 (LTBP-1) in association with TGF-beta 1 in ovarian carcinoma. *Jpn J Cancer Res* 92: 506-515, 2001.
2. Saito H, Tsujitani S, Oka S, Kondo A, Ikeguchi M, Maeta M and Kaibara N: An elevated serum level of transforming growth factor-beta 1 (TGF-beta 1) significantly correlated with lymph node metastasis and poor prognosis in patients with gastric carcinoma. *Anticancer Res* 20: 4489-4493, 2000.
3. Tsushima H, Kawata S, Tamura S, *et al*: High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology* 110: 375-382, 1996.
4. Bedossa P, Peltier E, Terris B, Franco D and Poynard T: Transforming growth factor-beta 1 (TGF-beta 1) and TGF-beta 1 receptors in normal, cirrhotic, and neoplastic human livers. *Hepatology* 21: 760-766, 1995.
5. Christeli E, Zoumpourlis V, Kiaris H, Ergazaki M, Vassilaros S and Spandidos DA: TGF- β 1 overexpression in breast cancer: correlation with clinicopathological data. *Oncol Rep* 3: 1115-1118, 1996.

6. Steiner MS and Barrack ER: Transforming growth factor-beta 1 overproduction in prostate cancer: effects on growth in vivo and in vitro. *Mol Endocrinol* 6: 15-25, 1992.
7. Krasagakis K, Tholke D, Farthmann B, Eberle J, Mansmann U and Orfanos CE: Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta2 in patients with disseminated malignant melanoma. *Br J Cancer* 77: 1492-1494, 1998.
8. Yamada N, Kato M, Yamashita H, Nister M, Miyazono K, Heldin CH and Funa K: Enhanced expression of transforming growth factor-beta and its type-I and type-II receptors in human glioblastoma. *Int J Cancer* 62: 386-392, 1995.
9. Takanami I, Imamura T, Hashizume T, Kikuchi K, Yamamoto Y and Kodaira S: Transforming growth factor beta 1 as a prognostic factor in pulmonary adenocarcinoma. *J Clin Pathol* 47: 1098-1100, 1994.
10. Tsushima H, Ito N, Tamura S, *et al.*: Circulating transforming growth factor beta 1 as a predictor of liver metastasis after resection in colorectal cancer. *Clin Cancer Res* 7: 1258-1262, 2001.
11. Friedman E, Gold LI, Klimstra D, Zeng ZS, Winawer S and Cohen A: High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. *Cancer Epidemiol Biomarkers Prev* 4: 549-554, 1995.
12. Langerak AD and Garewal HS: Transforming growth factor-beta 1: a useful tumor marker in patients with colorectal carcinoma? *Cancer* 85: 517-519, 1999.
13. Pardali K and Moustakas A: Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. *Biochim Biophys Acta* 1775: 21-62, 2007.
14. Hsu S, Huang F, Hafez M, Winawer S and Friedman E: Colon carcinoma cells switch their response to transforming growth factor beta 1 with tumor progression. *Cell Growth Differ* 5: 267-275, 1994.
15. Grady WM, Myeroff LL, Swinler SE, *et al.*: Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 59: 320-324, 1999.
16. Matsuzaki K, Seki T and Okazaki K: TGF-beta during human colorectal carcinogenesis: the shift from epithelial to mesenchymal signaling. *Inflammopharmacology* 14: 198-203, 2006.
17. Hauptmann S, Siegert A, Berger S, *et al.*: Regulation of cell growth and the expression of extracellular matrix proteins in colorectal adenocarcinoma: a fibroblast-tumor cell coculture model to study tumor-host interactions in vitro. *Eur J Cell Biol* 82: 1-8, 2003.
18. Hagedorn HG, Bachmeier BE and Nerlich AG: Synthesis and degradation of basement membranes and extracellular matrix and their regulation by TGF-beta in invasive carcinomas (Review). *Int J Oncol* 18: 669-681, 2001.
19. De Visser KE and Kast WM: Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia* 13: 1188-1199, 1999.
20. Siegert A, Denkert C, Leclerc A and Hauptmann S: Suppression of the reactive oxygen intermediates production of human macrophages by colorectal adenocarcinoma cell lines. *Immunology* 98: 551-556, 1999.
21. Derynck R and Zhang YE: Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425: 577-584, 2003.
22. Engel ME, McDonnell MA, Law BK and Moses HL: Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* 274: 37413-37420, 1999.
23. Yu L, Hebert MC and Zhang YE: TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *EMBO J* 21: 3749-3759, 2002.
24. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL and Arteaga CL: Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 275: 36803-36810, 2000.
25. Griswold-Prenner I, Kamibayashi C, Maruoka EM, Mumby MC and Derynck R: Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. *Mol Cell Biol* 18: 6595-6604, 1998.
26. Gibson SA and Shillito EJ: Ribozymes. Their functions and strategies for their use. *Mol Biotechnol* 7: 125-137, 1997.
27. Wichert A, Holm PS, Dietel M and Lage H: Selection of a high activity ribozyme against cytostatic drug resistance-associated glypican-3 using an in vitro assay containing total tumor RNA. *Cancer Gene Ther* 6: 263-270, 1999.
28. Anzano MA, Rieman D, Prichett W, Bowen-Pope DF and Greig R: Growth factor production by human colon carcinoma cell lines. *Cancer Res* 49: 2898-2904, 1989.
29. Lohr M, Schmidt C, Ringel J, Kluth M, Muller P, Nizze H and Jesnowski R: Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res* 61: 550-555, 2001.
30. Wu SP, Theodorescu D, Kerbel RS, Willson JK, Mulder KM, Humphrey LE and Brattain MG: TGF-beta 1 is an autocrine-negative growth regulator of human colon carcinoma FET cells in vivo as revealed by transfection of an antisense expression vector. *J Cell Biol* 116: 187-196, 1992.
31. Ashley DM, Kong FM, Bigner DD and Hale LP: Endogenous expression of transforming growth factor beta1 inhibits growth and tumorigenicity and enhances Fas-mediated apoptosis in a murine high-grade glioma model. *Cancer Res* 58: 302-309, 1998.
32. Huang F, Newman E, Theodorescu D, Kerbel RS and Friedman E: Transforming growth factor beta 1 (TGF beta 1) is an autocrine positive regulator of colon carcinoma U9 cells in vivo as shown by transfection of a TGF beta 1 antisense expression plasmid. *Cell Growth Differ* 6: 1635-1642, 1995.
33. Ye SC, Foster JM, Li W, *et al.*: Contextual effects of transforming growth factor beta on the tumorigenicity of human colon carcinoma cells. *Cancer Res* 59: 4725-4731, 1999.
34. Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR and Wakefield LM: TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 112: 1116-1124, 2003.
35. Wang D, Zhou GH, Birkenmeier TM, Gong J, Sun L and Brattain MG: Autocrine transforming growth factor beta 1 modulates the expression of integrin alpha 5 beta 1 in human colon carcinoma FET cells. *J Biol Chem* 270: 14154-14159, 1995.
36. Giannelli G, Fransvea E, Marinosci F, Bergamini C, Colucci S, Schiraldi O and Antonaci S: Transforming growth factor-beta1 triggers hepatocellular carcinoma invasiveness via alpha3beta1 integrin. *Am J Pathol* 161: 183-193, 2002.
37. Wojtowicz-Praga S: Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. *Invest New Drugs* 21: 21-32, 2003.
38. Lewis CE and Pollard JW: Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 66: 605-12, 2006.
39. Rabinovich GA, Gabrilovich D and Sotomayor EM: Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 25: 267-296, 2007.
40. Thomas DA and Massague J: TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 8: 369-380, 2005.
41. Matthews E, Yang T, Janulis L, Goodwin S, Kundu SD, Karpus WJ and Lee C: Down-regulation of TGF-beta1 production restores immunogenicity in prostate cancer cells. *Br J Cancer* 83: 519-525, 2000.
42. Adams DO and Hamilton DA: Macrophages as destructive cells in host defense. In: *Inflammation: Basic Principles and Clinical Correlates*. Galin JJ, Goldstein IM and Syndermann R (eds). Raven Press, New York, pp637-662, 1992.
43. Wu WS: The signaling mechanism of ROS in tumor progression. *Cancer Metastasis Rev* 25: 695-705, 2006.
44. Narai S, Watanabe M, Hasegawa H, Nishibori H, Endo T, Kubota T and Kitajima M: Significance of transforming growth factor beta1 as a new tumor marker for colorectal cancer. *Int J Cancer* 97: 508-511, 2002.