

## Evaluation of the interaction between TGF $\beta$ and nitric oxide in the mechanisms of progression of colon carcinoma

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Received 12 May 2005; accepted in revised form 30 June 2005

**Key words:** cancer progression, colon carcinoma, endothelium, macrophages, nitric oxide, TGF $\beta$

### Abstract

It is recognised that stromal cells determine cancer progression. We have previously shown that active TGF $\beta$  produced by rat colon carcinoma cells modulated NO production in rat endothelial cells. To elucidate the role of TGF $\beta$  and NO in the mechanisms of interaction of colon carcinoma cells with stromal cells and in cancer progression, we transfected REGb cells, a regressive colon carcinoma clone secreting latent TGF $\beta$ , with a cDNA encoding for a constitutively-secreted active TGF $\beta$ . Out of 20 injected rats only one tumour progressed, which was resected and sub-cultured (ReBeta cells). ReBeta cells secreted high levels of active TGF $\beta$ . The adhesive properties of REGb and ReBeta cells to endothelial cells were similar, showing that the secretion of active TGF $\beta$  is not involved in tumour cell adhesion to endothelial cells. ReBeta, but not REGb, cell culture supernatants inhibited cytokine-dependent NO secretion by endothelial cells, but inhibition of NO production was similar in co-cultures of REGb or ReBeta cells with endothelial cells. Therefore, secretion of active TGF $\beta$  regulated endothelial NO synthase activity when tumour cells were distant from, but not in direct contact with, endothelial cells. However, only ReBeta cells inhibited cytokine-dependent secretion of NO in coculture with macrophages, indicating that the active-TGF $\beta$ -NO axis confers an advantage for tumour cells in their interaction with macrophages rather than endothelial cells in cancer progression.

**Abbreviations:** BMDM – bone marrow-derived macrophages; CM – conditioned medium; DMEM – dulbecco's modified Eagle's medium; ELISA – enzyme-linked immunosorbent assay; FCS – fetal calf serum; IFN- $\gamma$  – interferon gamma; IL1 $\beta$  – interleukin-1beta; LPS – lipopolysaccharide; MMP – matrix metalloprotease; NO – nitric oxide; NOS – nitric oxide synthase; PBS – phosphate buffered saline; SD – standard deviation; TNF- $\alpha$  – tumour necrosis factor-alpha; TGF $\beta$  – transforming growth factor-beta

### Introduction

To progress and successfully metastasise tumour cells have to resist defence mechanisms of the host organ stromal cells. Nitric oxide (NO) derived from macrophages and endothelial cells has been shown to reduce adhesion of tumour cells to endothelial cells and participates in tumouricidal activity against many types of tumours [1–8]. Cells, including macrophages and endothelial cells, express an inducible form of NO synthase (iNOS) [9] which has been involved in defence mechanisms against pathogens and tumour cells. Consequently, the capacity of tumour cells to modulate the

level of NO production at the metastasis site could be crucial in regulating adhesive properties and survival of tumour cells and may determine the outcome of cancer metastasis.

TGF $\beta$  has been involved in the mechanisms regulating tumour progression. However, the function of TGF $\beta$  in cancer depends on the tumour stage and context [10]. In early stages TGF $\beta$  is anti-tumoural, inhibiting proliferation of tumour cells, whereas in later stages, TGF $\beta$  signalling pathways in tumour cells are inactivated. TGF $\beta$  then promotes cancer progression, acting as a permissive factor for stromal cells, inhibiting host immune surveillance, stimulating invasion, angiogenesis and metastasis. TGF $\beta$  also regulates protease secretion, including matrix metalloproteases (MMPs), or tumour cell interaction with endothelial cells [11–13]. We have previously shown that a progressive clone

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(PROb cells) of a chemically-induced rat colon carcinoma, secreting active TGF $\beta$  inhibited NO synthase induction and NO secretion in rat EC219 endothelial cells, whereas a regressive clone (REGb cells) isolated from the same colon carcinoma, secreting only latent TGF $\beta$  did not regulate NO production by EC219 cells [14]. In order to elucidate the role of tumour cell-derived TGF $\beta$  in the TGF $\beta$ -NO axis in cancer progression, we transfected REGb cells with a constitutively-secreted active form of TGF $\beta$  and evaluated the potential for these cells to form progressive tumours *in vivo* in tumour-cells syngeneic rats and to interact *in vitro* either with endothelial cells or macrophages expressing functional TGF $\beta$  receptors.

## Materials and methods

### Cell lines

The development and characterisation of the rat EC219 endothelial cell line have been described [15]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, 1 g/l glucose; Life Technologies, Basel, Switzerland) supplemented with 10% Fetal Calf Serum (FCS; Seromed, Fakola, Basel, Switzerland) and antibiotics on collagen type-I (Serva, Wallisellen, Switzerland) coated flasks. DHD/K12/TRs (referred to as REGb), a generous gift from Prof. F. Martin (INSERM, University of Dijon, France), is a sub-clone of the chemically induced rat colon carcinoma cell line DHD/K12. REGb cells form regressive tumours when inoculated into syngeneic BD-IX rats [16]. ReBeta cells are REGb cells which have been transfected with the cDNA encoding for active TGF $\beta$  and selected *in vivo* (see below). REGb and ReBeta cells were grown in 1:1 DMEM-HAM-F12 (Life Technologies) supplemented with 10% FCS and antibiotics. To prepare cell culture conditioned media (CM), cells were grown in culture media containing 10% FCS until reaching 75% confluence, then cultured for 48 h in serum-free culture medium. The culture supernatants were collected and spun for 10 min at 200  $\times$  g and the supernatants recovered. CCL-64 cells (a kind gift from Dr K. Frei, Department of Neurosurgery, University Hospital Zurich, Switzerland) is a mink-lung epithelial cell line whose proliferation is inhibited by TGF $\beta$ . CCL-64 cells were grown in DMEM supplemented with 5% FCS and 50  $\mu$ M  $\beta$ -mercaptoethanol (Sigma, Buchs, Switzerland).

### Isolation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were isolated from the femur and tibia of adult rats (OFA/SPF; BRL, Füllingsdorf, Switzerland). The bone-heads were cut and the bone marrow was collected by rinsing the bone cavity with 5 ml DMEM with a 25 G needle. Resulting cell suspension was spun at 100  $\times$  g for 5 min.

Cells were cultured in Sterilin petri dishes (Bibby Sterilin, Ostermundigen, Switzerland) in a differentiation culture medium consisting of 50% DMEM 1 g/l glucose, 30% culture supernatant of L929 mouse fibroblasts and 20% horse serum (Life Technologies) and antibiotics. After 7 days, cells were detached by incubation for 30 min at 4  $^{\circ}$ C and were further expanded in the differentiation culture medium for 5 days. BMDM were then washed with PBS and cultured in DMEM supplemented with 10% FCS for 24 h, before performing experiments.

### Transfection of REGb cells

Mutation of the Cys223 and Cys225 to Ser [17] induces the production of TGF $\beta$ 1 peptides which do not associate with the latency associated peptide resulting in the secretion of constitutively biologically active TGF $\beta$ 1. Five microgram of a PCRIII vector encoding mutated porcine TGF $\beta$ 1, or a void vector as control, in 50  $\mu$ l of 20 mM Hepes, pH 7.4 were adsorbed for 15 min at room temperature onto the DOTAP Liposomal Transfection Reagent (Roche Diagnostics, Rotkreuz, Switzerland) at 30  $\mu$ g/ml final concentration under the conditions described by the provider. Then 5 ml DMEM were added and the resulting suspension was added to half-confluent REGb cells. The cells were incubated for 4 h at 37  $^{\circ}$ C, washed and grown in DMEM containing 10% FCS for 5 days. Then transfected cells were selected for 2 weeks in the presence of 800  $\mu$ g/ml neomycin (Sigma), sub-cloned and grown in DMEM-HAM-F12 (1:1) containing 10% FCS. The production of active TGF $\beta$  was measured using the CCL-64 cell growth-inhibition assay (see below).

### Animal experimentation

Two clones of TGF $\beta$ -transfected REGb cells were selected (see Table 1) depending on their level of secretion of active TGF $\beta$  (determined using the CCL-64 cell growth-inhibition assay) and on their capacity to inhibit iNOS activity in EC219 cells. Parental or transfected REGb cells (transfected either with a void vector or the vector containing the cDNA encoding active TGF $\beta$ ) were injected subcutaneously ( $10^6$  cells in 500  $\mu$ l PBS per rat) into syngeneic BD-IX rats (10 rats/group; brother-sister mated since 1971 in the laboratory of Prof. F. Martin). Tumour growth was evaluated weekly for 10 weeks by measuring width and length of the tumour mass using calipers. The rats were then sacrificed. As previously described, REGb cells always regressed when injected under these conditions [16]. The only growing tumor arising from the injection of active TGF $\beta$ 1-transfected REGb cells was resected 10 weeks after injection, enzymatically digested to yield single cell suspension and expanded *in vitro* under the name ReBeta cells. Their secretion of active TGF $\beta$  was measured using the CCL-64 cell growth-inhibition assay and ELISA for active TGF $\beta$ .

Table 1. TGF $\beta$  secretion by colon carcinoma clones after transfection with constitutively-secreted active TGF $\beta$ .

	THF $\beta$ -transfected REGb clones				
	3	11	14	16	18
CCL-64 cell growth inhibition	++	+	+	+	+
iNOS inhibition in EC 219 cells					
Conditioned medium	+	-	+	-	+
Co-cultures	+	ND	+	ND	+

REGb transfected clones were grown to 75% confluence and culture medium was conditioned for 3 days in the absence of FCS. TGF $\beta$  content in the cell culture media was determined using either the CCL-64 growth inhibition assay, or the inhibition of NO secretion by EC219 cells or in co-cultures of transfected cells with endothelial cells.

++: High levels of active TGF $\beta$ ; +: medium levels of active TGF $\beta$ ; -: below detection limits; ND not determined.

#### *Inhibition of NO induction in EC219 cells*

These experiments were performed essentially as previously described [14]. Briefly, confluent EC219 cells were simultaneously exposed to 40 U/ml rat IFN- $\gamma$  (rIFN- $\gamma$ , Immunogenetics, Antwerpen, Belgium) and 200 U/ml murine TNF- $\alpha$  (mTNF- $\alpha$ , Roche), respectively and, when indicated, the culture supernatants of untransfected or transfected REGb cells for 24 h. Then NO secretion in the EC219 cell culture medium was quantified as nitrites using the Griess reagent as previously described [14]. To verify the induction of iNOS by cytokines in these cells, Western blotting experiments were performed using with an anti-mouse iNOS antibody (Transduction Laboratories, Basel, Switzerland).

#### *CCL-64 cell growth-inhibition assay for TGF $\beta$ quantification*

CCL-64 cells ( $5 \times 10^4$  per well) were incubated for 72 h at 37 °C in 96-well microtiter plates with serial dilutions of unknown samples or recombinant human TGF $\beta$ 1 (rhTGF $\beta$ ; R&D Systems, Abingdon, UK) at increasing concentrations (5 pg/ml to 1 ng/ml rhTGF $\beta$ ). Cell proliferation was assessed by addition of 250  $\mu$ g/ml MTT [3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) in DMEM during the last 2 h. MTT crystals were dissolved with 0.04 N HCl in isopropanol and optical density was measured at 540 nm in a multiwell microtiter plate reader (Labsystems iEMS Reader MF). TGF $\beta$ 1 activity (pg/ml) in the samples was determined by comparison with a rhTGF $\beta$  standard curve. Latent TGF $\beta$  was activated by heating the samples at 95 °C for 3 min, as previously described [14], then active peptide determined using the CCL-64 cells.

#### *Determination of TGF $\beta$ protein level by ELISA*

Secreted TGF $\beta$ 1 protein was measured in cell culture conditioned media using paired antibodies cross-reacting with rat and porcine TGF $\beta$ 1 (R&D Systems) according to the manufacturer's instructions. Ninety-six-well microtiter plates were coated with capture antibody (MAB 240; 4  $\mu$ g/ml in 100  $\mu$ l PBS) and

unknown or rhTGF $\beta$ 1 (5 pg/ml to 5 ng/ml rhTGF $\beta$ ) diluted in 20 mM Tris containing 150 mM NaCl, 0.1% BSA and 0.05% Tween 20 (diluent solution) were added and incubated for 2 h at room temperature, then biotinylated detection antibody (BAF 240; 50 ng/ml in diluent solution) was incubated for 2 h at room temperature. Quantification was done with peroxidase-coupled Streptavidin (62.5 ng/ml in diluent solution, Zymed Laboratories, San Francisco, CA), 0.006% H<sub>2</sub>O<sub>2</sub> and 100 mg/ml tetramethylbenzidine (Sigma) in 0.05 M phosphate-citrate buffer. The reaction was stopped with 0.5 M H<sub>2</sub>SO<sub>4</sub> and optical density was measured at 450 nm using a reference filter at 620 nm in a multiwell microtiter plate reader (Labsystems). Concentrations of TGF $\beta$ 1 in samples were calculated by comparison with a standard curve using rhTGF $\beta$ .

#### *NO and TGF $\beta$ secretion by REGb and ReBeta cells under cytokine stimulation*

Cells were exposed to 40 U/ml rIFN- $\gamma$ , 200 U/ml mTNF- $\alpha$  and 500 pg/ml rat IL-1 $\beta$  (rIL-1 $\beta$ , R&D system, Abingdon, UK) for 24 h and NO or TGF $\beta$  secretion in the cell culture medium was quantified as nitrites using the Griess reagent as previously described [14], or using the CCL-64 cell growth inhibition assay, for NO or TGF $\beta$ , respectively.

#### *Co-cultures of tumour cells with endothelial cells or macrophages*

EC219 endothelial cells were plated alone or together with either REGb or ReBeta tumour cells in 48-well plates ( $2.5 \times 10^4$  EC219 cells and/or  $5 \times 10^4$  tumour cells per well). Cells were allowed to adhere overnight at 37 °C and stimulated with 40 U/ml rIFN- $\gamma$  plus 200 U/ml mTNF- $\alpha$  for 24 h in complete culture medium. Culture supernatants were collected and nitrite concentrations determined using the Griess reagent [14]. Bone marrow-derived macrophages were plated in 48-well plates 24 h prior to addition of REGb or ReBeta tumour cells ( $5 \times 10^4$  cells per well). After 24 h, cells were activated with 40 U/ml rIFN- $\gamma$  plus 100 ng/ml Lipopolysaccharide (LPS; Sigma) for 24 h in the presence of

10% FCS. Culture supernatants were collected and nitrite concentrations determined. Cultures of EC219, REGb or ReBeta cells or macrophages alone were used as controls.

#### Adhesion assay

EC219 cells in 48-well plates were either untreated or treated for 48 h with either 40 U/ml rIFN- $\gamma$  plus 100 U/ml mTNF- $\alpha$  or with 4 and 2 ng/ml or 400 pg/ml rhTGF $\beta$ . Cells were washed twice with DMEM without FCS immediately prior to adhesion assay. REGb and ReBeta tumour cells were labelled with 400 nCi/ml [ $^3$ H]thymidine (Amersham, Aylesbury, UK) for 10 h and detached for 1 h at 37 °C with EDTA (Versene, Life Technologies). REGb or ReBeta tumour cells ( $10^5$  cells per well) were added to the EC219 cells in DMEM without FCS. After 30 min at 37 °C, non-adherent cells were removed, collected and spun for 10 min at  $200 \times g$ . Adherent and non-adherent cells were dissolved overnight in 0.1% SDS/0.1 M NaOH and radioactivity was quantified in a  $\beta$  counter (LKB Rackbeta, Helsinki, Finland).

#### Zymogram analysis of gelatinase activity

REGb-CM and ReBeta-CM supernatants were prepared as described above and concentrated 30-fold by centrifugation through a 5 kDa cut-off polymer membrane (Millipore AG, Volketswil, Switzerland). Samples (25  $\mu$ g/lane) were resolved without prior denaturation by electrophoresis in 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Merck, Switzerland). As control, recombinant human MMP-2 (rhMMP-2; 4 ng/lane; Oncogene Research Products, Luzern, Switzerland) was run in parallel. Following electrophoresis, gels were washed free of SDS with 2.5% Triton X-100 (Fluka, Buchs, Switzerland) and incubated at 37 °C for 24 h in 50 mM Tris-HCl pH 7.5 containing 200 mM NaCl, 5 mM CaCl $_2$  and 0.02% Brij-35 (Fluka) and stained in with 0.05% Coomassie Brilliant Blue G-250 (Serva). MMP-2 activity was identified by its comigration with recombinant human MMP-2.

#### Statistical evaluation

Means, standard deviations (SD) and statistical significance were calculated using a paired Student's *t*-test.

## Results

Rat REGb colon carcinoma cells were transfected either with the cDNA encoding constitutively-secreted active TGF $\beta$ 1 or the empty vector as control. Transfected cells grew at the same rate than parental cells. Following 2 week selection and sub-cloning, constitutive secretion of active TGF $\beta$  was measured in the culture supernatants of five different clones using the CCL-64 cell

growth-inhibition assay, the inhibition of NO secretion by endothelial EC219 cells or by co-cultures of transfected REGb cells with endothelial cells (Table 1). Clone 3 produced very high levels of active TGF $\beta$ , as assessed by strong inhibition of CCL-64 cell growth and of NO secretion by EC219 cells and were rejected for animal experimentation to avoid risks of fibrosis. Clones 11 and 16 produced low levels of active TGF $\beta$  using the CCL-64 cell growth-inhibition assay and their conditioned media did not inhibit NO secretion by EC219 cells. Clones 14 and 18 secreted levels of active TGF $\beta$  that were able to inhibit CCL-64 cell growth and NO secretion by EC219 cells. Therefore these 2 clones were selected for further animal experimentation. Either REGb cells, REGb transfected with the empty vector (REGb control), clone 14 or clone 18 were sub-cutaneously (s.c.) injected into syngeneic rats. Injection of parental REGb cells, confirming previous results [16], or injection of cells transfected with the empty vector, always resulted in regressive tumours within 30 days. Out of 20 rats injected with clone 14 or clone 18, only one tumour developed spontaneously from clone 14 (Figure 1). This tumour was resected, recultured under the name ReBeta cells and characterised.

The secretion of total (latent + active) TGF $\beta$  by ReBeta and REGb cells was measured using an ELISA (Table 2). As previously described [14], REGb cells secreted nearly detectable levels of active TGF $\beta$  whereas ReBeta cells secreted very high levels of TGF $\beta$ . Active TGF $\beta$  secretion under cytokine stimulation was also determined using the CCL-64-growth inhibition assay (Figure 2). rIFN- $\gamma$  alone did not modulate TGF $\beta$  secretion either by REGb or ReBeta cells. rIFN- $\gamma$  in combination with mTNF- $\alpha$  did not modulate TGF $\beta$  secretion by REGb cells, but increased TGF $\beta$  secretion by ReBeta cells (Figure 2). The secretion of NO by REGb and ReBeta cells upon cytokine stimulation was also evaluated. Addition of rIFN- $\gamma$  and mTNF- $\alpha$

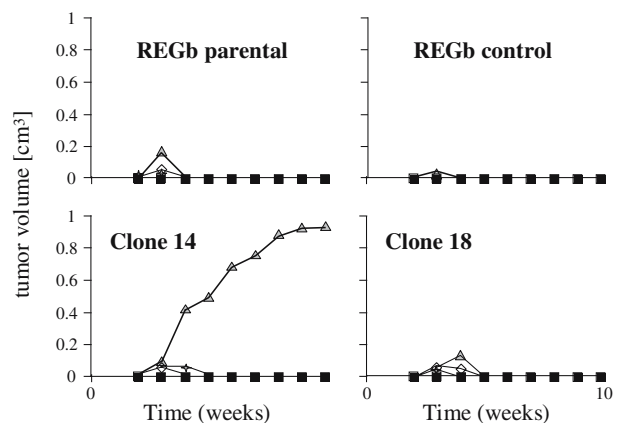


Figure 1. *In vivo* progression of REGb cells and cell clones. REGb cells ( $10^6$  cells per rat), either parental (REGb parental), or mock-transfected (REGb control), or transfected with constitutively-secreted active TGF $\beta$  (clone 14 and clone 18) were injected subcutaneously in syngeneic BD-IX rats (10 rats per group). Tumour volume (in cm $^3$ ) was evaluated weekly for 10 weeks.

Table 2. ELISA determination of secretion of total (active + latent) TGFβ by REGb and ReBeta cells.

Conditioned media of tumor cells	TGFβ (pg/ml)
REGb-CM	44 ± 10
ReBeta-CM	2926 ± 50

Cells were grown to 75% confluence and culture medium was conditioned for 2 days in the absence of FCS. TGFβ secretion in the cell culture media was determined by a specific ELISA, and concentrations of TGFβ were calculated by comparison with rhTGFβ as a standard. Values are the means of triplicate determinations ± SD.

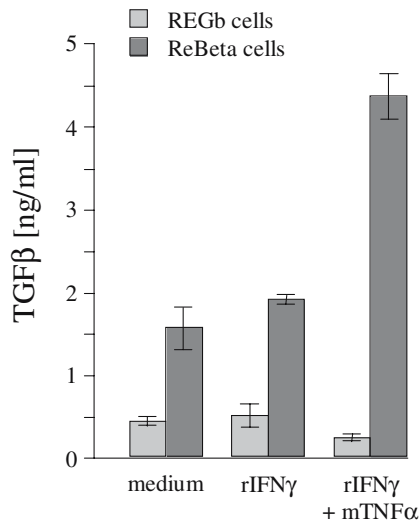


Figure 2. Secretion of TGFβ by cytokine-stimulated REGb and ReBeta cells. REGb (light grey bars) or ReBeta (dark grey bars) cells were grown to 60% confluence, and either not stimulated (medium) or stimulated with the indicated cytokines (40 U/ml rIFN-γ, 100 U/ml mTNF-α) for 48 h and active TGFβ secretion was quantified using the CCL-64 growth inhibition assay. Data are means of triplicate wells ± SD of one representative experiment out of 3.

resulted in a slight increase of NO secretion only by REGb cells, and the combination of rIFN-γ, mTNF-α and IL-1β resulted in a significant increase of NO secretion by both cell lines ( $P < 0.01$ ) although the cytokine-dependent increase of NO secretion by ReBeta cells was significantly lower than by REGb cells ( $P < 0.01$ ) (Figure 3). ReBeta and REGb cells secreted equivalent amounts of matrix metalloproteinase (MMP)-2, while secretion of MMP-9 was absent in both cells (Figure 4).

The effects of factors secreted by REGb and ReBeta cells on the secretion of NO by EC219 endothelial cells was evaluated. Treatment of EC219 endothelial cells with 40 U/ml rIFN-γ and 100 U/ml mTNF-α for 24 h induced NO release (Figure 5), involving up-regulation of iNOS expression as determined by mRNA level or iNOS activity [14] or by Western Blot (Figure 5, insert). Addition of ReBeta-cell conditioned medium (ReBeta-CM) to EC219 cells simultaneously with cytokines inhibited NO secretion whereas REGb-cell conditioned medium (REGb-CM) did not (Figure 5). Then the role of tumour cell-secretion of TGFβ on rapid adhesion of

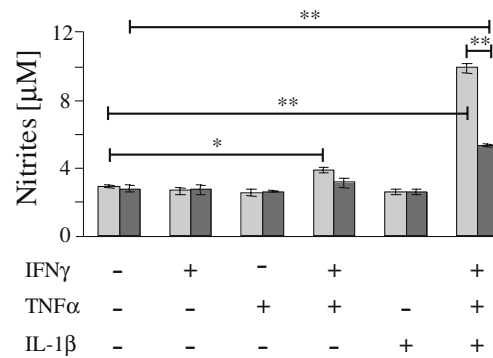


Figure 3. NO production by REGb and ReBeta cells under cytokine stimulation. REGb (light grey bars) or ReBeta (dark grey bars) cells were grown to 60% confluence, and either not stimulated (-) or stimulated (+) with the indicated cytokines (40 U/ml rIFN-γ, 100 U/ml mTNF-α, 500 pg/ml rIL-1β) for 48 h and NO secretion was determined. Statistical significance was assessed using a two-tailed paired Student's *t*-test, \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ .



Figure 4. MMP-2 secretion by REGb and ReBeta cells. REGb and ReBeta cells were grown to sub-confluence and their culture media was conditioned for 2 days in the absence of FCS, concentrated and 25 μg protein per lane were subjected to zymography. Recombinant MMP-2 was used as a standard.

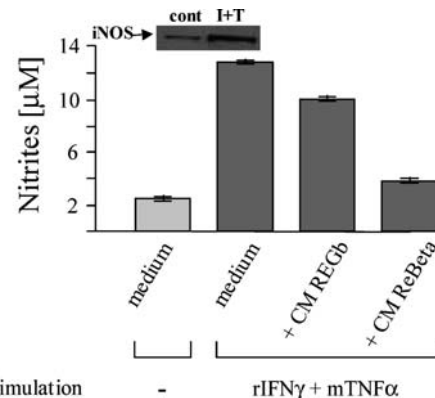
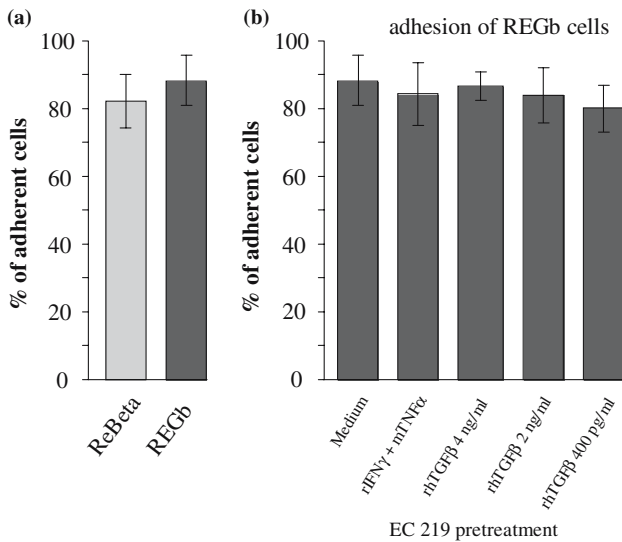


Figure 5. Effects of soluble factors secreted by REGb or ReBeta cells on NO production by endothelial cells. EC219 endothelial cells were grown to 60% confluence, and either not stimulated (medium, light grey bar) or stimulated with 40 U/ml IFN-γ and 100 U/ml TNF-α, in the absence (medium, dark grey bar) or the presence of either REGb (CM REGb, 50% final concentration) or ReBeta conditioned medium (CM ReBeta, 50% final concentration) for 24 h and NO secretion was determined. Data are means of triplicate wells ± SD of one representative experiment out of 3. Insert: The induction of iNOS protein by cytokine stimulation of EC219 cells was verified by Western blotting.

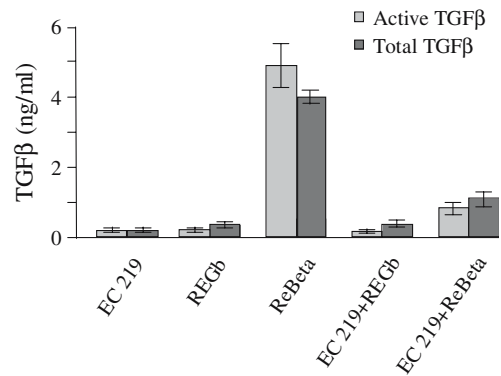
<sup>3</sup>H-Thymidine-labelled REGb or ReBeta cells to monolayers of endothelial cells was evaluated. REGb and ReBeta cells adhered similarly to EC219 cells (Figure 6a). Treatment of endothelial cells with 40 U/ml



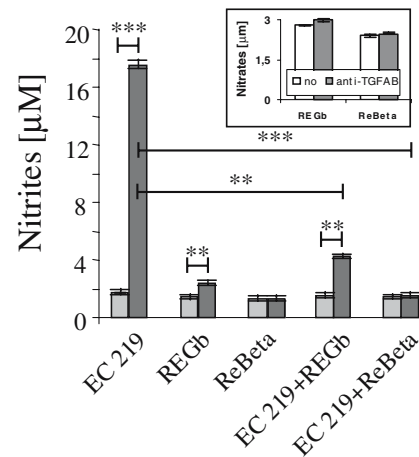
**Figure 6.** Adhesion of REGb or ReBeta cells to endothelial cells. (a) Unstimulated EC219 cells were exposed to  $^3\text{H}$ -Thymidine-labelled REGb or ReBeta cells for 30 min, and adherent tumour cells were quantified. (b) EC219 cells were stimulated with 40 U/ml IFN- $\gamma$  and 100 U/ml TNF- $\alpha$ , or with rhTGF $\beta$  for 48 h, then exposed for 30 min to  $^3\text{H}$ -Thymidine-labelled REGb or ReBeta (results not shown) cells, and adherent tumour cells were quantified. Results are means  $\pm$  SD of 3 independent experiments.

rIFN- $\gamma$  plus 100 U/ml mTNF- $\alpha$  or with increasing concentrations of rhTGF $\beta$  (400 pg/ml up to 4 ng/ml rhTGF $\beta$ ) for 48 h prior to adhesion assay did not modify their adhesive properties for either REGb (Figure 6b) or ReBeta cells (data not shown). In order to eliminate endothelial cell line-specific effects we performed similar experiments using another rat endothelial cell line, the CR3 cells [18], with similar information (results not shown). Next, the effect of direct contact between REGb or ReBeta cells with endothelial cells on the secretion of active or total TGF $\beta$  was quantified in the supernatant of co-cultures of EC219 cells with either REGb or ReBeta cells. EC219 cells did not secrete TGF $\beta$ , no active TGF $\beta$  and only low levels of latent TGF $\beta$  were detected in the supernatants of co-cultures of EC219 with REGb cells, whereas the secretion of active TGF $\beta$  in co-cultures of ReBeta cells with EC219 cells was decreased (Figure 7).

Finally the effect on NO secretion of direct contact between REGb or ReBeta cells and EC219 endothelial cells or bone marrow-derived macrophages was evaluated. Co-culture of EC219 endothelial cells with either REGb or ReBeta tumour cells did not modulate NO production in the absence of added cytokines (results not shown). However, NO production was inhibited when cytokine-stimulated EC219 cells were in co-cultures with either REGb or ReBeta cells (Figure 8). The inhibition of cytokine-induced NO production by EC219 cells can be reversed by addition of a blocking anti-TGF $\beta$ 1 antibody [14] demonstrating the role of TGF $\beta$  in the inhibition of NO secretion by EC219 cells. Addition of blocking anti-TGF $\beta$ 1 antibody to the co-cultures did not restore NO secretion by cytokine-

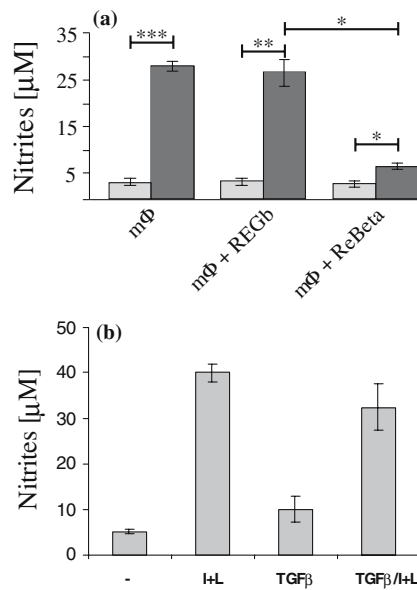


**Figure 7.** TGF $\beta$  secretion by co-cultures of endothelial cells and REGb or ReBeta cells. EC219, REGb or ReBeta cells were grown either alone (EC219, REGb, ReBeta) or in co-culture (EC219 + REGb, EC219 + ReBeta) for 24 h, then stimulated with 40 U/ml IFN- $\gamma$  and 100 U/ml TNF- $\alpha$  for 24 h, and active (light grey bars) and total (dark grey bars) TGF $\beta$  secretion was quantified using the CCL64 cell assay in the cell culture supernatants. Data are means of triplicate wells  $\pm$  SD of one representative experiment out of 3.



**Figure 8.** NO production in co-cultures of endothelial cells with either REGb or ReBeta cells. EC219, REGb or ReBeta cells were grown either alone (EC219, REGb, ReBeta) or in co-culture (EC219 + REGb, EC219 + ReBeta) for 24 h, then either untreated (light grey bars), or stimulated with 40 U/ml IFN- $\gamma$  and 100 U/ml TNF- $\alpha$  (dark grey bars) for 24 h, and NO secretion was determined. Statistical significance was assessed using a two-tailed paired Student's *t*-test, \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ . Results are means of duplicate wells  $\pm$  SD of one representative experiment out of 3. Insert: In co-cultures of EC219 cells with either REGb (REGb) or ReBeta (ReBeta) cells, addition of an anti-TGF $\beta$ -blocking antibody did not restore NO secretion in response to cytokines. White bars: cytokines; grey bars: cytokines + anti-TGF $\beta$  antibody.

stimulated EC219 cells (Figure 8, insert). Activation of macrophages with rIFN- $\gamma$  and LPS resulted in the secretion of large amounts of NO (Figure 9a). When macrophages were in contact with REGb cells, NO secretion was not modified, however in contact with ReBeta cells, NO secretion was inhibited, suggesting that the levels of active TGF $\beta$  secretion by colon carcinoma cells is involved in the regulation of production of NO by macrophage, but not by endothelial cells. Pre-



**Figure 9.** NO production by co-cultures of macrophages with either REGb or ReBeta cells. (a) Macrophages (m $\Phi$ ) were grown alone, or with either REGb or ReBeta (m $\Phi$  + REGb, m $\Phi$  + ReBeta) cells for 24 h, then either untreated (light grey bars) or treated with 40 U/ml IFN- $\gamma$  and 100 ng/ml LPS for 24 h, and NO concentrations were measured in culture supernatants. Results are means  $\pm$ SD of triplicate wells of one representative experiment out of 3. Statistical significance was assessed using a two-tailed paired Student's *t*-test, \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ . (b) Macrophages were either untreated or pre-treated for 24 h with 1.5 ng/ml rhTGF $\beta$  (TGF $\beta$  bars), then exposed either to culture medium (-, TGF $\beta$ ) or stimulated with 40 U/ml IFN- $\gamma$  and 100 ng/ml LPS for 24 h (I + L, TGF $\beta$ /I + L), and NO concentrations were measured in culture supernatants. Results are means  $\pm$ SD of triplicate wells of one representative experiment out of 3.

treatment of macrophages for 24 h prior to rIFN- $\gamma$  and LPS stimulation was not able to inhibit their NO secretion (Figure 9b).

## Discussion

TGF $\beta$  family ligands signal through a common mechanism bringing together type I and type II Ser/Thr kinase receptors, resulting in the phosphorylation of type I receptor by type II receptor and activation of intracellular signalling pathways. Colon cancer cells predominantly produce TGF $\beta$  in a latent form [19]. TGF $\beta$  is secreted as a latent complex of 290 kDa consisting of bioactive TGF $\beta$  (Mr 25 kDa), the latency-associated peptide (LAP; Mr 75 kDa), and the latent TGF $\beta$  binding protein 1 (LTBP1; Mr 190 kDa). LTBP1 is attached to LAP by a disulfide bond, which is required for the assembly and secretion of the latent TGF $\beta$  complex and prevents TGF $\beta$  signalling [20]. Mutations of Cys223 and Cys225 to Ser in the propeptide region of TGF $\beta$ 1 precursor [17] result in TGF $\beta$ 1 peptides which do not associate to the LAP, and secretion of constitutively biologically active TGF $\beta$ 1. TGF $\beta$  has been proposed to favour tumour progression in colon carcinoma but its contribution has not been clearly established. TGF $\beta$  is a

potent growth inhibitor for normal epithelial cells, whereas in carcinoma cells, including colon cancer cells, mutations in the genes encoding TGF $\beta$  receptors frequently inactivate signalling [21]. TGF $\beta$  produced by tumour cells also exerts a paracrine effect on tumour stroma [12]. Therefore in the early stages of cancer TGF $\beta$  is anti-tumoural, inhibiting proliferation of tumour cells, whereas in later stages, TGF $\beta$  rather promotes cancer progression by acting as a permissive factors for stromal cells to inhibit host immune surveillance, and stimulate invasion, angiogenesis and metastasis.

In the present experiments, in order to address the role of the interplay between TGF $\beta$  and NO in cancer progression, we tested the hypothesis that the levels of active-TGF $\beta$  secretion by tumour cells is involved in stromal cell functions, particularly in modulating tumour cell interaction with endothelial cells and macrophages. To evaluate this hypothesis, we decided to perform *in vivo* selection of REGb cells engineered to secrete high levels of active TGF $\beta$ , then to perform *in vitro* studies, comparing REGb cells with active-TGF $\beta$ -transfected REGb cells which had demonstrated the capacity for *in vivo* progression. Therefore REGb cells were transfected with a cDNA encoding constitutively-secreted active TGF $\beta$  and these modified REGb cells were injected in syngeneic rats. The *in vivo* progression of tumour cells was evaluated and the interaction *in vitro* of a progressive tumour, ReBeta cells, with endothelial cells and macrophages was analysed in relation with tumour cell-dependent secretion of TGF $\beta$  and regulation of NO production.

Our results demonstrated that the secretion by colon carcinoma cells of high levels of active TGF $\beta$  is not sufficient for a high incidence of tumour progression since out of 20 animals only one harboured a progressive tumour, which after resection still secreted high levels of TGF $\beta$ . With the present approach we cannot ascertain whether the expression of the transfected peptide was maintained in the 19 animals which did not develop tumours. The autocrine secretion of high levels of active TGF $\beta$  did not inhibit growth of TGF $\beta$ -transfected REGb cells, suggesting the loss of responsiveness of these cells for this effect of TGF $\beta$ . After *in vivo* progression of TGF $\beta$ -transfected REGb cells, cytokine activation further increased their TGF $\beta$  secretion, but decreased their production of NO compared to REGb cells. Levels of active TGF $\beta$  secretion in this model did not modify MMP-2 production, therefore excluding a role for TGF $\beta$  in promoting cancer progression via this pathway, which is in accordance with the information that tumour-associated fibroblasts are the main source of MMPs in tumours. NO has been shown to increase tumour cells adhesion to cytokine-stimulated endothelial cells [3], or to reduce tumour cell adhesion to post-capillary venules [6]. The cytokines TNF- $\alpha$  and IFN- $\gamma$  are known to increase adhesive properties of the endothelium. Treatment of endothelial cells with recombinant TGF $\beta$  or exposure of endothelial cells to

melanoma cells secreting latent TGF $\beta$  enhanced adhesion of tumour cells to endothelial cells [22]. Our present results demonstrated that the levels of active TGF $\beta$  secretion by colon carcinoma cells and the modulation by TGF $\beta$  of NO secretion by endothelial cells did not modify adhesive properties between endothelial and tumour cells, irrespective of cytokine stimulation of endothelial cells. In our model, the absence of modulation of tumour cells adhesion to cytokine-activated endothelial cells suggested that the interactions of colon carcinoma cells with endothelial cells are not based on the modulation by cytokines of the adhesive properties of adhesion molecules. In agreement with these results we have previously shown in a breast cancer model of lung metastasis that NO did not influence the early stages of tumour metastasis and the interaction of invading tumour cells with pulmonary endothelial cells, but that the cellular origin of NO was critical to the metastatic process [23]. Progressive colon carcinoma cells (PROb cells) secreting active TGF $\beta$  had the ability to inhibit cytokine-induced NO production in EC219 endothelial cells and microglial cells while a regressive clone (REGb cells) isolated from the same carcinoma but secreting only latent TGF $\beta$  did not display these properties [14]. In PROb cells, regulation of NO secretion by TGF $\beta$  was an intracrine process, whereas exogenously added TGF $\beta$  was ineffective [24].

The activation of latent TGF $\beta$  has been shown to be mediated by direct contact between endothelial and smooth muscle cells [25]. In co-cultures of EC219 with REGb or ReBeta cells, both cell lines behaved comparably, suggesting that latent TGF $\beta$  could be activated through intercellular mechanisms. Tumour-cell derived IFN- $\beta$  stimulates NO production by host cells, including macrophages, and suppresses tumour progression [26]. TGF $\beta$  is able to block cytokine-mediated activation of macrophages and their NO secretion [27, 28] and is involved in the suppression of local immune response and NO-mediated macrophage cytotoxicity. Therefore tumour progression was inversely related to NO secretion by macrophages [29]. In our model, the level of TGF $\beta$  secretion, and the resulting modulation of NO secretion by macrophages was important in the mechanisms of interaction of colon carcinoma cells with macrophages. The decreased secretion of NO could be due to the blockade of macrophage activation by high levels of active TGF $\beta$  secreted by ReBeta cells, but not REGb cells. However, pre-exposure of macrophages to rhTGF $\beta$  before rIFN- $\gamma$  and LPS stimulation failed to mimic this inhibitory effect. These results suggest that continuous presence of high levels of TGF $\beta$ , as achieved in co-culture of macrophages with ReBeta cells, is necessary for this effect, or that direct contact, or alternatively that other molecules, are involved.

In conclusion, our study demonstrates that the secretion of high levels of active TGF $\beta$  is not sufficient to generally allow colon carcinoma cells to progress *in vivo*. These results also demonstrate that the adhesion of tumour cells to endothelial cells does not depend on the

level of secretion of active TGF $\beta$  by tumour cells, suggesting that the NO-modulated adhesion of tumour cells to the endothelium is not a limiting factor for progression. The inhibition of NO production in macrophages depended on the level of active TGF $\beta$  secreted by tumour cells, indicating that immunomodulation of the host defence mechanisms by high TGF $\beta$  and the resulting modulation of the TGF $\beta$ -NO axis may be related to tumour progression.

### Acknowledgements

The authors thank the Swiss League against Cancer (Grants SKL 353-9-1996, KFS 947-09-1999 and KPS 1070-09-2000), the program "Co-tutelle de thèse" of the University of Lausanne and EPHE, the Programme d'Actions Intégrées Germaine de Stael and the Swiss Society for Multiple Sclerosis, for supporting these researches.

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