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# TNFα Down-regulates CD105 Expression in Vascular Endothelial Cells: A Comparative Study with TGFß1

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**Abstract.** *The vascular endothelium participates in angiogenesis, inflammation and the immune response, which are modulated by vasoactive cytokines such as tumour necrosis factor-α (TNFα) and transforming growth factor-ß1 (TGFß1). CD105 is a component of the TGFß receptor complex and is abundantly expressed in activated/injured endothelium where it is implicated in multiple cellular processes. Up-regulation of CD105 in synovial cells of rheumatoid arthritis and psoriatic lesions implies a possible role in the pathogenesis of such inflammatory disorders. The pro-inflammatory cytokine, TNFα, and anti-inflammatory cytokine, TGFß1, regulate multiple cellular processes such as proliferation, differentiation and apoptosis. Our hypothesis is that CD105 gene expression in endothelial cells is regulated by the multifunctional cytokines TNFα and TGFß1. By using human dermal microvascular endothelial cells the present study has shown that long-term treatment with TNFα (0.1-5 ng/ml) elicited a concentration- and time-dependent significant suppression (over 50% reduction) in CD105 protein levels. The observations that no significant alterations in the CD105 mRNA levels or in the CD105 promoter activity were found and that the potent inhibitor of NFκB, PDTC, did not affect the TNFα action suggest that CD105 down-regulation by TNFα is not at the transcriptional level. In contrast to TNFα, TGFß1 significantly elevated CD105 protein and mRNA expression (<2-fold increase) through activation of its promoter activity. From these data we conclude that TNFα and TGFß1 exert opposing effects on CD105 expression in human vascular endothelial cells and that CD105 is enmeshed in the network of signal pathways modulating multiple cellular functions.*

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The vascular endothelium participates in angiogenesis, inflammation and the immune response. Cytokines such as tumour necrosis factor-α (TNFα) and transforming growth factor-ß1 (TGFß1) modulate these processes partially by acting on their vascular targets such as vascular endothelial growth factor (VEGF) and its receptor KDR (1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (2-4). CD105 (endoglin) is an important component in the TGFß receptor complex that is expressed mainly in vascular endothelial cells (EC) (5,6) and to a lesser extent in macrophages (7), erythroid precursors (8), syncytiotrophoblast in placenta (9), pre-B leukaemic (10), haematopoietic (11), mesangial (12) and vascular smooth muscle cells (13), implying its multifunctional potential. CD105 binds TGFß1, TGFß3, activin-A and BMP-7 and modulates TGFß signalling (14-16). Haploin-sufficiency of CD105 results in hereditary haemorrhagic telangiectasia (HHT) type I - an autosomal dominant vascular disorder (17;18). CD105 knock-out mice develop severe defects in their vasculature and die *in utero*. Suppression of CD105 *in vitro* leads to inhibition of angiogenesis (19-22). The expression of CD105 in vascular endothelial cells of tumours is much more intense than in normal tissues and correlates with tumour metastasis and poor prognosis (23-28). Overexpression of CD105 has also been observed in the synovial cells of rheumatoid arthritis (RA), psoriatic lesions and in the skin vasculature of patients with systemic scleroderma, suggesting that it may be important in the pathogenesis of the inflammatory auto-immune disorders (29,30 and our unpublished data).

TGFß, a multifunctional cytokine, modulates angiogenesis and suppresses inflammation and the immune response (31,32). With regard to its role in tumour development, published literature is contradictory - TGFß1 either inhibits or promotes tumour progression depending on the stage of the disease (31,33). TGFß signalling can be modified by CD105 on the one hand, while on the other hand CD105 is up-regulated by TGFß in cultured human monocytes (15). There appears to be a mutually dependent regulation

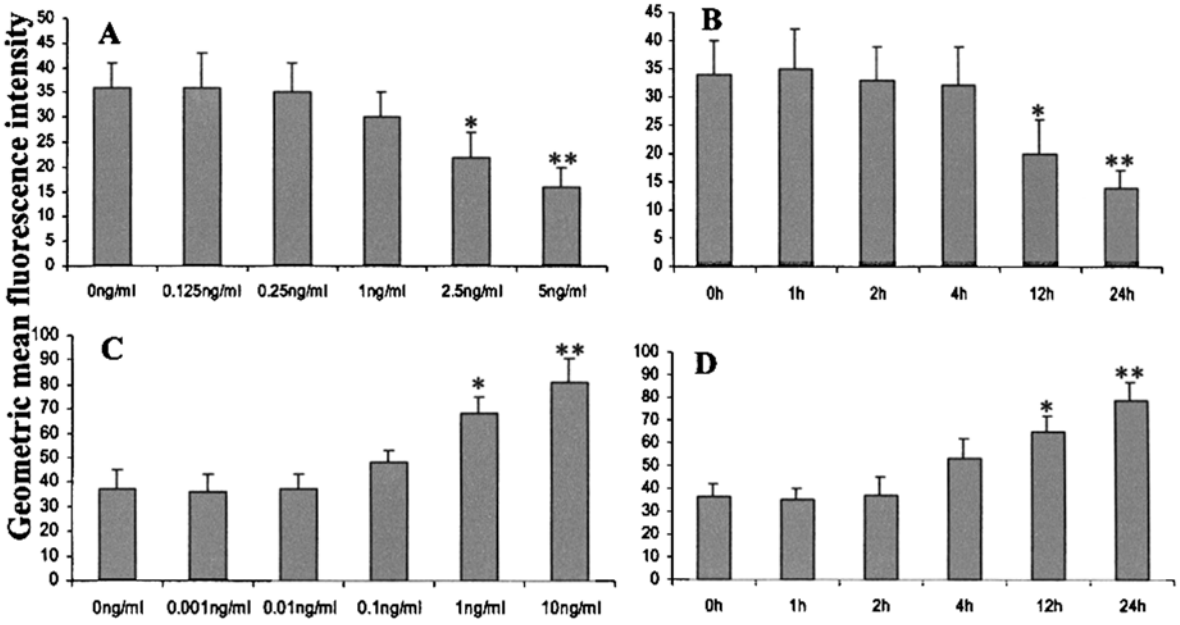


Figure 1. *FACS analysis of CD105 expression. HDMEC were cultured in medium containing TNFα or TGFβ1 for 24 hours and CD105 expression was quantified by FACS. (A) TNFα induced a concentration-dependent reduction of CD105 expression with the maximal effects occurred at 5 ng/ml. (B) HDMEC were treated with 5 ng/ml of TNFα for various times. TNFα did not significantly alter CD105 levels from 1 to 4 hours, but it did so from 12 to 24 hours of treatment. (C) Treatment with TGFβ1 led to a concentration-dependent increase in CD105 expression with the maximal effect occurring at 10 ng/ml. (D) HDMEC were cultured in medium containing 10 ng/ml of TGFβ1 for designated times. The cells responded to TGFβ1 in a typical time-dependent manner, with treatment for 24 hours eliciting maximal CD105 expression. Data were expressed as mean±s.e.m.. Each bar represents data collected from six separate experiments (*p<0.05 and **p<0.01 compared with the control as analysed by one-way ANOVA followed by Duncan test).*

between CD105 and TGFß1 in CD105 expressing cells. To ascertain whether this is the case in human vascular EC, the effect of TGFß1 on the expression of CD105 in human dermal microvascular EC (HDMEC) was examined.

TNFα, a pro-inflammatory cytokine, is produced upon stimulation by monocytes, macrophages, T and B lymphocytes, neutrophils and mast cells and is involved in multiple cellular functions including proliferation, differentiation and apoptosis (34). *In vivo* TNFα induces extensive disruption of tumour vasculature followed by haemorrhagic tumour necrosis (35,36). The anti-vascular action of TNFα is highly selective as it has no detectable effect on quiescent vessels (37,38). However, the molecular basis for the selectivity of TNFα's action on angiogenic blood vessels has not been fully elucidated. Recently TNFα has been reported to suppress the activation of αvß3 (36). With regard to inflammatory diseases, TNFα plays a major role in the pathogenesis of RA and psoriasis (39,40), wherein the concomitant presence of CD105 has been noted. Accordingly, in this study the effect of TNFα on the expression of CD105 in HDMEC was examined.

## Materials and Methods

*HDMEC and cell culture.* HDMEC (Clonetics, San Diego, CA, USA) were grown in complete medium comprising MCDB 131, 10 ng/ml epithelial growth factor, 10% (v/v) foetal calf serum (FCS) (Life Technologies), 2 mM glutamine plus 100 μg/ml penicillin and 100 μg/ml streptomycin. Confluent cells were subcultured using 0.05% (w/v) trypsin and 2 mM EDTA. These cells express high levels of CD105 and are also positive for pan-endothelial markers, *viz* CD31 and von Willebrand factor as determined by immunocytochemistry, flow cytometry and immunoblotting (data not shown). To study the gene expression of CD105 in response to cytokines, confluent HDMEC were maintained in complete medium, containing either TNFα (0.125 - 5 ng/ml) (Sigma) or TGFß1 (0.001 - 10 ng/ml) (R&D Systems) for up to 24 hours. Control cells at the same confluency were maintained in complete medium only (lacking TNFα and TGFß1). The cultures were rinsed twice with pre-warmed PBS to remove any detached cells prior to examination of the following parameters. The possible effect of pyrrolidinedithiocarbamate (PDTC) (Sigma), a potent NFκB inhibitor, on the action of TNFα was examined using serum-free cultures. Fifty μM of PDTC were added to the HDMEC in the presence or absence of TNFα for 24 hours and CD105 expression was determined by immunoblotting analysis.

*Indirect immunofluorescence and flow cytometric analysis.* The cell surface expression of CD105 protein was quantified by flow cytometry as described previously (21). Briefly, $10^5$ cells per tube were incubated with 50 μl (10 μg/ml in PBS) of monoclonal antibody (mab), E9, to CD105 or pre-immunised mouse serum as negative control antibody (10 μg/ml in PBS) on ice for 1 hour and washed twice with cold PBS. After incubation with FITC-labelled rabbit anti-mouse $F(ab)_2$ (1:40; DAKO) for 30 minutes on ice, the cells were washed and re-suspended in 0.3 ml of 2% buffered formalin and analysed on a Becton Dickinson FACScan flow cytometer.

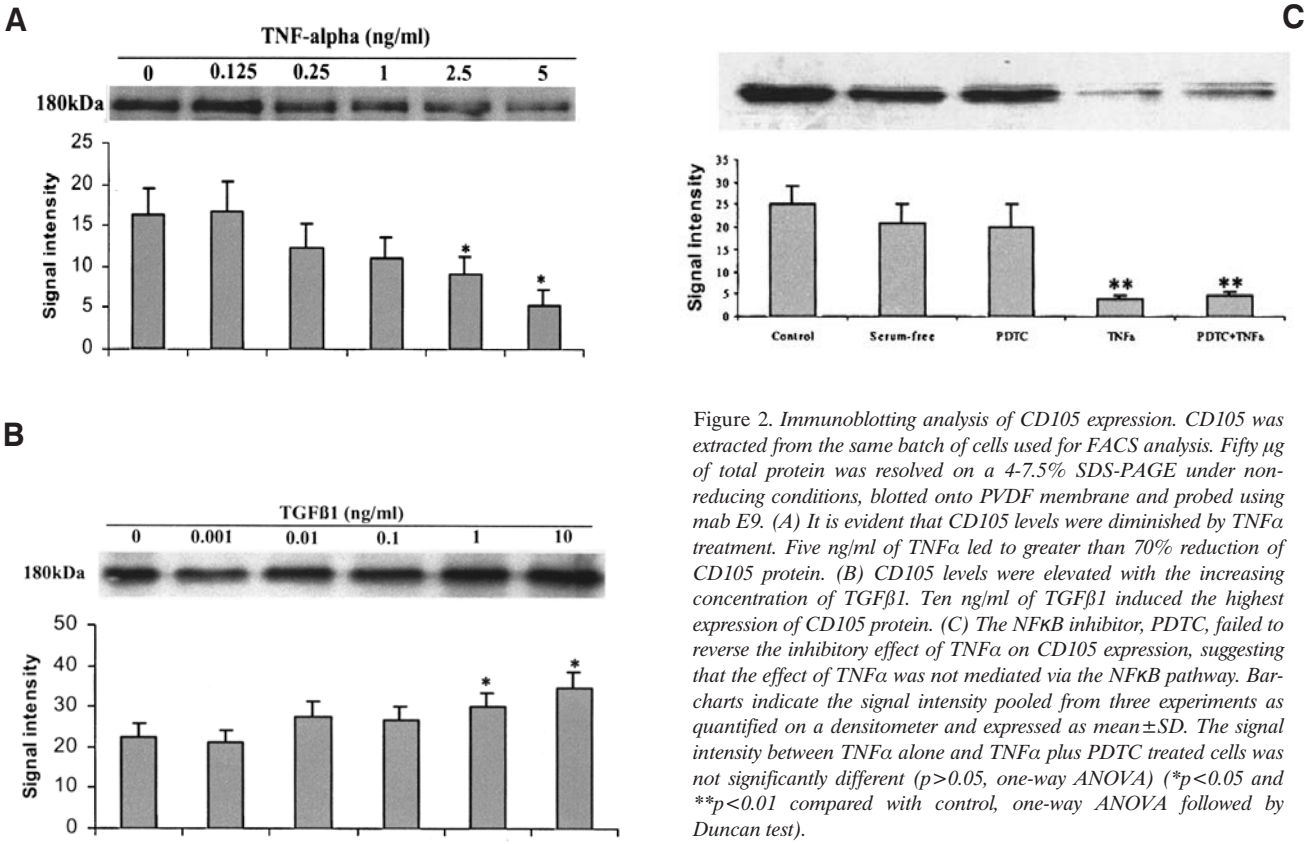


Figure 2. *Immunoblotting analysis of CD105 expression. CD105 was extracted from the same batch of cells used for FACS analysis. Fifty μg of total protein was resolved on a 4-7.5% SDS-PAGE under non-reducing conditions, blotted onto PVDF membrane and probed using mab E9. (A) It is evident that CD105 levels were diminished by TNFα treatment. Five ng/ml of TNFα led to greater than 70% reduction of CD105 protein. (B) CD105 levels were elevated with the increasing concentration of TGFβ1. Ten ng/ml of TGFβ1 induced the highest expression of CD105 protein. (C) The NFκB inhibitor, PDTC, failed to reverse the inhibitory effect of TNFα on CD105 expression, suggesting that the effect of TNFα was not mediated via the NFκB pathway. Bar-charts indicate the signal intensity pooled from three experiments as quantified on a densitometer and expressed as mean±SD. The signal intensity between TNFα alone and TNFα plus PDTC treated cells was not significantly different ($p>0.05$, one-way ANOVA) (*$p<0.05$ and **$p<0.01$ compared with control, one-way ANOVA followed by Duncan test).*

*Analysis of CD105 protein expression by immunoblotting.* CD105 protein was extracted from HDMEC by solubilising $1\times10^7$ cells/ml with extraction buffer [0.2% (v/v) NP-40 in 0.1 M Tris buffer (pH 7.3), 0.5 mM PMSF, 1 mM pepstatin, 0.1 mM leupeptin, 1 mM EDTA (BDH)]. The cell lysate was microfuged at 8000×g for 10 minutes at 4˚C and the supernatant collected for immunoblotting analysis. Cell lysate corresponding to 50 μg of protein was added to an equal volume of sample buffer [0.1 M Tris-HCl, 4% (w/v) SDS, 0.001% (w/v) bromophenol blue, 20% (w/v) glycerol (BDH)] and resolved on 4-7.5% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel. The fractionated proteins were electrophoretically transferred onto a PVDF membrane (Millipore) using the Trans-Blot system (Bio-Rad Laboratories). Filters were blocked with 2% (w/v) BSA in PBS, 0.1% (v/v) Tween 20 for 2 hours at room temperature. To detect CD105 protein, mab E9 to CD105 (1:1000; 0.5 μg/ml) in blocking solution was applied and filters were incubated overnight at 4˚C. Finally, the blots were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (DAKO) (1:2000 in blocking solution) for 2 hours at 4˚C with shaking. The CD105 protein was visualised using the enhanced chemiluminescence (ECL) system (Amersham). All chemicals unless specified were obtained from Sigma.

*Analysis of CD105 mRNA by Northern blotting.* Total RNA was extracted using guanidinium thiocyanate/phenol/chloroform. Briefly, HDMEC cultured in 25-$cm^2$ flasks were lysed *in situ* by the addition of 1 ml RNAzol B (Biogenesis). The cell lysate was transferred to clean Corex tubes, to which 0.2 ml of chloroform was added and mixed by inversion. Following centrifugation at 8000×g for 15 minutes at 4˚C, the upper phase was removed to a clean Corex tube, and an equal volume of isopropanol added to precipitate the RNA. The RNA was recovered by centrifugation (8000×g) at 4˚C for 15 minutes and the pellet was washed in 70% ethanol and air-dried. The samples were resuspended in 50 μl DEPC $H_2O$ on ice and the amount of RNA was quantified by measuring $OD_{260}$.

For Northern blot analysis, 20 μg of total RNA was denatured and fractionated in 1% (w/v) agarose / 2.8% (v/v) formaldehyde gel and capillary blotted onto nitrocellulose. The CD105 cDNA probe was excised with Eco RI from the pcEXV-EndoL plasmid (15) and the 2.3 kb fragment was labelled with $^{32}P$ by random prime labelling. The blot was hybridised with the $^{32}P$-labelled cDNA probe complementary to CD105 mRNA at 65˚C overnight and the extent of probe hybridisation was revealed by a phosphorimager (Molecular Dynamics), and analysed using Image Quant software. The blots were then re-hybridised with $^{32}P$-labelled probe for GAPDH for use as a loading control.

*Luciferase reporter gene assay.* To determine the CD105 promoter activity following TNFα or TGFß1 treatment, plasmid pXP2 harbouring the 2.8-kb (-2450/+350) CD105 promoter (41) and a down-stream firefly luciferase gene was used for transient transfection of HDMEC. Internal normalisation was performed by co-transfection of the pXP2 plasmid with CMVßgal, a ß-galactosidase expression vector driven by the cytomegalovirus (CMV) promoter. Transfection of HDMEC was carried out using the liposome-mediated gene transfer technique. Briefly, cells were seeded at $1\times10^5$ cells/35-mm dish and the following day were transfected with 4 μl of DMRIE-C (Life Technologies) plus 1μg of plasmid CMVßgal mixed with 2 μg of pXP2 in serum-free medium. Twenty-four hours after transfection, the cultures were replenished with complete medium with or without TNFα or TGFß1 and incubated for 24 hours. Thereafter the cells were harvested and the enzyme activity determined. Luciferase and ß-galactosidase activities were measured on

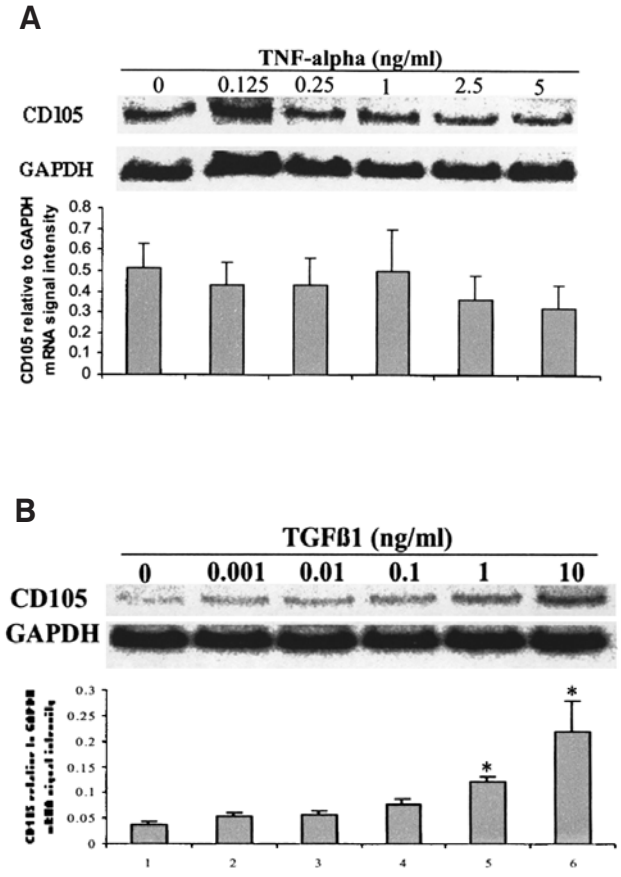


Figure 3. *Northern blotting analysis of CD105 mRNA expression. Twenty micrograms of total RNA were fractionated on a 1% denaturing agarose gel and transferred onto a nylon membrane. CD105 and GAPDH were probed using $^{32}$P-labelled corresponding cDNAs and visualised on a Phosphorimager. (A) Treatment with TNFα resulted in an insignificant alteration of CD105 mRNA (p>0.05, one-way ANOVA). (B) Treatment with TGFβ1 elevated CD105 mRNA in a typical concentration-dependent manner. In agreement with FACS and immunoblotting analysis, 10 ng/ml of TGFβ1 elicited the strongest signal for CD105 mRNA. Bar charts indicate the signal intensity pooled from three experiments and expressed as mean±SD. (*p<0.05, one-way ANOVA followed by Duncan test).*

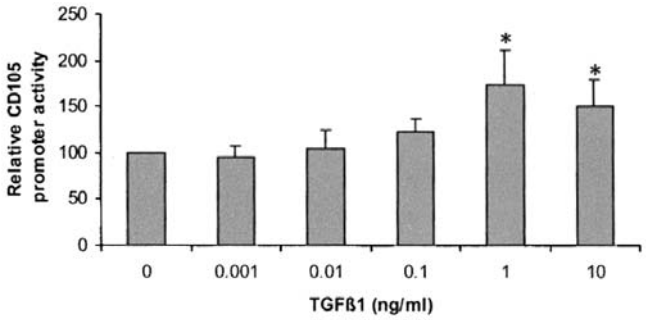


Figure 4. *CD105 promoter activity upon treatment with TGFβ1. HDMEC were co-transfected with plasmids pXP2/pCD105/lux and CMVβgal and cultured in the presence or absence of TGFβ1. Luciferase and β-galactosidase activities were determined 24 hours post-treatment. To eliminate possible variations in cell number and transfection efficiency, luciferase was normalised to β-galactosidase activity. TGFβ1 treatment significantly elevated the CD105 promoter activity, which was most evident at 1 and 10 ng/ml. Data represent six samples at each concentration and expressed as mean±s.e.m., collected from three separate experiments (*p<0.05 compared with control medium, one-way ANOVA followed by Duncan test).*

a TD-20/20 Luminometer (Promega, UK) and a colorimetric plate reader (Labsystems) respectively using kits from Promega.

## Results

*TNFα suppressed but TGFβ1 increased expression of CD105 as determined by FACS.* HDMEC were grown in medium containing TNFα (0, 0.125, 0.25, 0.5, 2.5 and 5 ng/ml) or TGFß1 (0, 0.001, 0.01, 0.1, 1 and 10 ng/ml) for 24 hours. Cells were harvested and stained with mab E9 to quantify the cell surface expression of CD105 by FACS. TNFα suppressed CD105 expression in a concentration-dependent manner. Five ng/ml of TNFα resulted in a maximal reduction in CD105 protein levels (approximately 50%) (Figure 1A). Therefore in subsequent experiments, HDMEC were treated with this concentration of TNFα (5 ng/ml) for various time courses. A typical time-dependent response was noted, with maximal suppression occurring at 24 hours, but with significant effects taking place after 12 hours (Figure 1B).

The addition of TGFß1 resulted in an increased expression of CD105 - the highest expression (approximately 100% increase) was elicited by 10 ng/ml (Figure 1C). To examine whether cellular responses to TGFß1 are time-dependent, cells were grown in medium supplemented with 10 ng/ml of TGFß1 for various times. Longer incubation resulted in higher CD105 expression, demonstrating that TGFß1 up-regulated CD105 expression in a time-dependent manner (Figure 1D).

*Immunoblotting analysis of CD105 expression.* HDMEC were treated with various concentrations of TNFα and TGFß1 separately for 24 hours and cell lysate corresponding to 50 μg protein was subjected to SDS-PAGE under non-reducing conditions, blotted onto PVDF membrane and probed using mab E9. In all cases one major band of 180 kDa corresponding to the dimeric form of CD105 was seen on the blot. As shown in Figure 2A, CD105 levels correlated inversely with TNFα concentration - for instance, 5 μg/ml of TNFα reduced CD105 levels by approximately 70%. In contrast, TGFß1 elevated CD105 expression in a concentration-dependent manner. Ten ng/ml of TGFß1 induced the maximal CD105 expression in comparison with

the control medium (Figure 2B). To investigate whether inhibition of the NFκB affected TNFα regulation on CD105 expression, cells grown in serum-free medium were treated with 50 μM PDTC for 24 hours in the presence or absence of TNFα. As shown in Figure 2C, TNFα but not PDTC markedly reduced CD105 levels. The addition of PDTC to TNFα did not alter the effect of the latter, demonstrating that the TNFα regulation was not mediated by the NFκB pathway. It is also evident that cells cultured in serum-free medium expressed considerably lower levels of CD105 than those in medium supplemented with 10% FCS, which is in line with the data obtained by FACS (data not shown).

*Northern blot of CD105 mRNA from cells following treatment with TNFα or TGFβ1.* Total RNA extracted from TNFα or TGFß1-treated or untreated control cells was fractionated in a denaturing agarose gel, blotted onto nitrocellulose membrane and probed using cDNAs to CD105 and GAPDH. Treatment with TNFα resulted in a negligible reduction of CD105 mRNA in comparison to GAPDH mRNA, suggesting that the suppression of CD105 expression by TNFα is not at the transcriptional level (Figure 3A). The following result from luciferase reporter gene assay was in favour of this observation. As shown in Figure 3B, TGFß1 induced a typical concentration-dependent increase in CD105 mRNA expression. The expression patterns of mRNA in TGFß1-treated cells were consistent with the protein levels revealed by FACS and immunoblotting.

*TGFβ1 but not TNFα markedly altered CD105 promoter activity.* To examine whether TNFα and TGFß1 exert their effects on the CD105 promoter, HDMEC were transiently transfected with the plasmid pXP2 harbouring a CD105 promoter and a down-stream firefly luciferase gene. Treatment with TNFα resulted in a negligible suppression of CD105 promoter activity (data not shown), which was considered not strong enough to support the transcriptional regulation. In contrast, upon treatment with TGFß1, CD105 promoter activity was elevated (over 1.5-fold compared with the basal level), demonstrating that TGFß1 up-regulates CD105 expression through enhancing its promoter activity (Figure 4).

## Discussion

TNFα and TGFß1 are involved in a number of patho-physiological settings including angiogenesis, tumour development, inflammation and immune response. CD105 plays a critical role in maintaining the normal structure and physiological function of blood vessels and promoting formation of the neovasculature. The data presented in this study have revealed an association between CD105 and the multifuntional proinflammatory cytokine, TNFα, and the anti-inflammatory cytokine, TGFß1. The implication of these findings is that CD105 is one of the vascular targets for the two cytokines and modulates their actions on the vascular system.

The differential regulation of CD105 by TNFα and TGFß1 in EC is another example of the two cytokines displaying contradictory functions. For instance, TNFα and TGFß1 exhibit opposing effects on the expression of cell cycle proteins such as p21, p27 and cyclin D1 (42), connective tissue growth factor (43), and matrix metalloproteinase 9 in monocytes (44). VCAM-1 is one of the indicators of endothelial damage/activation (2;4). TNFα stimulates the expression of VCAM-1 but this response is repressed by TGFß1 in glomerular EC, again indicating the distinct opposing actions of these two cytokines (45).

TGFß1 is generally regarded as playing a dual role in tumour development, *i.e.* it reduces tumourigenicity of breast and lung cancers by suppressing tumour growth and angiogenesis (46,47) but may promote progression of an advanced tumour through immune suppression and indirect stimulation of angiogenesis (33). A number of investigators have reported that over-expression of TGFß1 in tumour tissues and in the circulation of patients with late stage cancers of prostate, stomach, colorectum and lung is associated with the occurrence of metastases and shorter survival (48-50). VEGF has been reported to mediate the positive effects of TGFß1 in tumour angiogenesis (51). The present study indicates that CD105 may be another mediator of the action of TGFß1 on angiogenesis in advanced tumours. CD105 is over-expressed in tumour tissues and in the circulation of patients with various types of cancer, Its expression levels correlate with angiogenesis, metastasis and poor prognosis (24-26,52,53). Data presented in this study demonstrate that TGFß1 up-regulates CD105 expression in vascular EC. The latter, in turn, may promote angiogenesis. Therefore we speculate that the stimulatory action of TGFß1 on CD105 gene expression contributes to the worsening prognosis of certain types of advanced cancers. Further studies are needed to clarify the possible inter-relationship between TGFß1, CD105, angiogenesis and tumour progression in the same cohort of patients.

TNFα has been used for anti-cancer therapy, its target being the vascular endothelium of the vasculature and not the tumour cells. Administration of TNFα in patients with metastatic melanoma resulted in detachment and apoptosis of EC (35). The inactivation of integrin αvß3 by TNFα is one of the contributing factors, but other mechanisms may be involved in the anti-angiogenic action of TNFα (36). In view of the putative pro-angiogenic role of CD105, the observed inhibition of CD105 in EC by TNFα may be another important contribution to its suppressive effect on tumour angiogenesis.

Both TNFα and TGFß1 are present in atherosclerotic lesions and are thought to have key roles in atherogenesis (54,55). Clinical and experimental evidence suggest that development of atherosclerotic lesions, which is initiated by lipoprotein retention and aggregation within the vessel wall,

can be accelerated by the local action of inflammatory cytokines such as TNFα on EC. TNFα stimulates expression of EC genes like VCAM-1 and E-selectin that may exacerbate the progression of atherosclerosis (45,55). In contrast, TGFß1 is considered to exert atheroprotective effects through inhibition of smooth muscle cell migration and suppression of inflammation (56,57). CD105 is present in atherosclerotic plaques and elevated levels are found in the circulation of patients with atherosclerosis (13,57 and our unpublished data), but its role in the atherogenic processes remains to be established.

CD105 is up-regulated in inflammatory conditions such as RA, psoriasis and tissue repair (29,30,59,60). The results shown here suggest that the pro-inflammatory factor TNFα does not contribute to this CD105 up-regulation. It remains possible that under different experimental conditions, TNFα has a positive effect on CD105 expression. TNFα plays a major role in the progression of inflammatory diseases such as RA, psoriasis as demonstrated by the success of anti-TNFα therapy in RA (58). However, it is not known what effect anti-TNFα therapy has on CD105 levels. Quantification of CD105 in diseased tissues and in the circulation of patients pre- and post-treatment may prove whether it is an appropriate marker of disease activity.

Systemic sclerosis (SSc) is an autoimmune disease characterised by the involvement of microvascular damage, excessive extracellular molecule deposition and fibrosis. In an unpublished study we have found up-regulation of CD105 in the vasculature of skin biopsies taken from patients with SSc. Further investigation is required to determine the interactions of TGFßs and CD105 in the pathobiology of this disease.

The fact that TGFß1 but not TNFα significantly altered CD105 promoter activity suggests that the TGFß1 regulation resides, at least in part, at the transcriptional level. The stimulatory effect of TGFß1 has been previously observed in mesangial and CD105-transfected U937 cells (15); these findings are in agreement with the present study. Several transcriptional elements responsive to TGFß, including the Smad binding elements found in the CD105 promoter, are considered to mediate the effect of TGFß1 on CD105 expression (41, 61). By contrast, the CD105 down-regulation by TNFα, shown in this paper, does not support a transcriptional involvement. We speculate that under our experimental conditions, TNFα may induce cleavage of CD105 by an unknown protease, which requires further investigation.

The findings in this study, together with the observation that IFNγ, a potent anti-angiogenic cytokine, also suppresses CD105 gene expression in vascular EC (our unpublished data) lead us to conclude that CD105 expression in the EC is regulated by the concerted action of a panel of vasoactive cytokines. As a pro-angiogenic factor, CD105 modulates their actions on the vasculature. In the context of the multifunctional property of such cytokines, CD105 appears to be embedded in a network of signals involved in angiogenesis, inflammation and immune response. Further studies are required to elucidate the precise roles of CD105 in these processes and interactions of CD105 with other intracellular components.

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