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CD105 prevents apoptosis in hypoxic endothelial cells

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Summary

CD105, a marker of endothelial cells, is abundantly expressed in tissues undergoing angiogenesis and is a receptor for transforming growth factor β. The pivotal role of CD105 in the vascular system was demonstrated by the severe vascular defects that occur in CD105-knockout mice, but the exact mechanisms for CD105 regulation of vascular development have not been fully elucidated. In light of the function of CD105 and the importance of hypoxia in neovascularisation, we speculated that CD105 is involved in hypoxia-initiated angiogenesis. Using tissue-cultured human microvascular endothelial cells, we have investigated the effects of hypoxic stress on CD105 gene expression. Hypoxia induced a significant increase in membrane-bound and secreted CD105 protein levels. CD105 mRNA and promoter activity were also markedly elevated, the latter returning to the basal level after 16 hours of hypoxic stress. Hypoxia induced cell cycle arrest at the G0/G1 phases and massive cell apoptosis after 24 hours through a reduction in the Bcl-2 to Bax ratio, downregulation of Bcl-X_L and Mcl-1, and upregulation of caspase-3 and caspase-8. The consequence of CD105 upregulation was revealed using an antisense approach and a TUNEL assay. Suppression of CD105 increased cell apoptosis under hypoxic stress in the absence of TGFβ1. Furthermore, hypoxia and TGFβ1 synergistically induced apoptosis in the CD105-deficient cells but not in the control cells. We conclude that hypoxia is a potent stimulus for CD105 gene expression in vascular endothelial cells, which in turn attenuates cell apoptosis and thus contributes to angiogenesis.

Key words: Hypoxia, CD105, Endothelial cells, TGFβ1, Apoptosis

Introduction

Angiogenesis, the formation of neovasculature, is important for tumour growth and metastasis, diabetic retinopathy, rheumatoid arthritis and atherosclerosis (Folkman, 1995; Kumar et al., 1996). CD105, a receptor for TGFβ1 and TGFβ3, is a crucial molecule in vascular development and angiogenesis. Overexpression of CD105 is found in neoplastic and regenerating tissues where it is implicated in promoting angiogenesis (Burrows et al., 1995; Seon and Kumar, 2001; Takahashi et al., 2001; van de Kerkhof et al., 1998; Wikstrom et al., 2002; Tanaka et al., 2001). Targeted disruption of the CD105 gene in mice leads to defective embryonic angiogenesis and foetal death, which indicates that it is an absolute requirement for normal vascular development (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). CD105 gene mutations in man are responsible for hereditary haemorrhagic telangiectasia type 1, an autosomal dominant disorder characterised by multisystemic vascular dysplasia and recurrent haemorrhage (Shovlin and Letarte, 1999). We and others have shown that CD105 forms complexes with the TGFβ receptors I and II and actively regulates TGFβ signalling in human vascular endothelial cells (ECs) and CD105-transfected cells (Lastres et al., 1996; Li et al., 2000a; Yamashita et al., 1994). TGFβ regulates cell proliferation, differentiation, extracellular matrix metabolism, angiogenesis and vascular integrity (Pepper, 1997). CD105 appears to function in these processes by regulating TGFβ signalling.

Hypoxia is a common feature of tumour and ischaemic tissues (Semenza, 2000). If the cellular oxygen concentration fails to match the requirements of energy metabolism, a number of genes are triggered to respond to the hypoxic environment. Hypoxia-inducible genes promote cell survival by expediting oxygen delivery to the oxygen-deprived tissues (e.g. erythropoietin) (Wanner et al., 2000), by increasing glucose transport (e.g. glucose transporter-1) (Ebert et al., 1995), by raising the levels of glycolytic enzymes (e.g. lactate dehydrogenase A) (Kambe et al., 1998) and, most importantly, by promoting angiogenesis (Marti and Risau, 1999; Yue and Tomanek, 1999). A number of angiogenic factors, such as vascular endothelial growth factor (VEGF) (Mukhopadhyay et al., 1995; Schweiki et al., 1992), angiopoietin-2 (Mandriota et al., 2000), erythropoietin (Huang et al., 1997) and fibroblast growth factor (Sakaki et al., 1995) are induced by oxygen-deprivation to facilitate angiogenesis.

Considering the pro-angiogenic function of CD105, we speculated that there might be an association between oxygen deprivation and CD105 gene expression. Should such a relationship exist, it would not only explain the augmented expression of CD105 in tumours and ischaemic tissues but would also have therapeutic implications for many angiogenic diseases. Furthermore, elucidating the function of CD105

under hypoxic stress would improve our understanding of the pathogenesis of several vascular diseases in which CD105 is implicated.

Materials and Methods

Cell culture

Human dermal microvascular endothelial cells (HDMECs) (Clonetics, San Diego, CA) were grown in complete growth medium composed of MCDB 131, 10 ng/ml epithelial growth factor, 10% (v/v) foetal calf serum (Invitrogen), 2 mM glutamine plus 100 μg/ml penicillin and 100 μg/ml streptomycin. For hypoxic exposure, confluent HDMECs were incubated within a modular incubator chamber filled with a gas mixture of 0.2% oxygen, 5% carbon dioxide and 94.8% nitrogen placed in a 37°C incubator. Control cells with the same confluency were maintained at normoxic conditions (20% oxygen, 5% carbon dioxide and 75% nitrogen) in the same incubator. To study cell apoptosis under hypoxic stress, HDMECs in complete growth medium were cultured in the hypoxic chamber in the presence or absence of recombinant human TGFβ1 (R&D Systems, Abingdon, UK) for 24 hours. We used ELISAs that were sensitive for TGFβ1 and TGFβ3 [and can detect as little as 20 pg/ml of TGFβ1 and 100 pg/ml of TGFß3 (Li et al., 2000b)], but we did not detect TGFβ1 or TGFβ3 in the complete growth medium. However, in order to eliminate any trace amount of TGFβ1 and TGFβ3 in the medium, neutralising antibodies to TGFβ1 and TGFβ3 were administered in parallel experiments. Ten μg/ml of chick anti-TGFβ1 (R&D Systems) and an equal amount of goat anti-TGFβ3 (R&D Systems) were added to the cultures in the absence of exogenously added TGFβ1 or TGFβ3 during exposure to hypoxia. This antibody concentration was sufficient to neutralise the bioactivity of 2,000 pg/ml of TGFβ1 and 250 pg/ml of TGFβ3.

To suppress CD105 expression in HDMECs, an antisense approach was applied as described previously (Li et al., 2000a). Briefly, the 16-mer antisense phosphorothioate-modified oligodeoxynucleotide (AS ODN: 5′-ATGCTGTCCACGTGGG-3′) and the scrambled control ODN with the same base composition (SC ODN: 5′-ACTCGTGC-TACGGTGG-3′) were synthesised (Applied Biosystems, Foster City, CA). Subconfluent HDMECs grown in 35 mm petri dishes were transfected with 0.5 nM/ml of AS or SC ODN plus 8 μg/ml of DMRIE-C (Invitrogen) in serum-free medium. After overnight incubation, the medium was replenished with complete growth medium, and incubation was continued for an additional 48 hours in normoxia. Thereafter the medium was replaced with fresh complete growth medium, and the cultures were exposed to hypoxia for up to 24 hours.

Analysis of CD105 protein expression

Cell surface expression of CD105 protein in AS-, SC-treated or untreated cells was quantified by flow cytometry as described previously (Li et al., 2000a). Briefly, 10^5 cells per tube were incubated with 50 μl (10 μg/ml in PBS) of anti-CD105 mAb E9 or pre-immunised mouse serum as a negative control antibody (10 μg/ml in PBS) on ice for 1 hour and washed twice with cold PBS. mAb 44G4 to human CD105 (Gougos and Letarte, 1988) was also employed for comparison with mAb E9. After incubation with a fluorescein-labelled $F(ab')_2$ fragment of rabbit anti-mouse antibody (1/40; DAKO, Denmark) 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.

For analysis of CD105 protein by immunoblotting, CD105 protein was extracted from HDMECs by solubilising 1×10^7 cells/ml with a cocktail buffer [0.2% (v/v) Nonidet P-40 in 0.1 M Tris buffer (pH 7.3), 0.5 M PMSF, 1 mM pepstatin, 0.1 mM leupeptin, 1 mM EDTA] and resolved on 4-7.5% (w/v) sodium dodecyl sulphate (SDS)-polyacrylamide gel and electrophoretically transferred onto a PVDF membrane (Millipore) (Li et al., 2000a). mAb E9 (1:1000; 0.5 μg/ml) in blocking solution was applied to detect CD105 protein, and filters were incubated overnight at 4°C. Finally, the blots were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (DAKO) for 2 hours at 4°C with shaking. The CD105 protein was visualised using the enhanced chemiluminescence system (Amersham Biosciences, UK). As an internal loading control, the blot was stripped and reprobed using a goat anti-α-actin (Sigma) or mouse anti-CD31 antibody (DAKO) and detected by HRP-conjugated secondary antibodies. CD105 shed into the medium was detected by a chemiluminescent ELISA system as described previously (Wang et al., 1995; Li et al., 2000c).

Analysis of CD105 mRNA by northern blotting

Total RNA from HDMECs was extracted using guanidinium thiocyanate/phenol/chloroform (Chomczynski and Sacchi, 1987). RNA samples (15 μg) were denatured and fractionated in 1% (w/v) agarose/2.8% (v/v) formaldehyde gel and blotted onto nitrocellulose. The CD105 probe used was a 2.3 kb fragment excised with *Eco*RI from the pcEXV-EndoL plasmid (Lastres et al., 1996) and labelled with ^{32}P by random prime labelling. The blot was hybridised with the ^{32}P-labelled probe and the hybridisation was revealed by a phosphorimager (Molecular Dynamics), then analysed using Image Quant software. The blots were rehybridised with ^{32}P-labelled probe for GAPDH for use as a loading control.

Luciferase reporter gene assay

To determine the level of CD105 promoter activity, plasmid pXP2 harbouring the 2.8 kb (–2450/+350) CD105 promoter (Rius et al., 1998) and a downstream firefly luciferase gene was used for transient transfection of HDMECs. Internal normalisation was performed by cotransfection of the pXP2 plasmid with CMVβgal, a β-galactosidase expression vector driven by the cytomegalovirus (CMV) promoter. Transfection of HDMECs was carried out using the liposome-mediated gene transfer technique. Briefly, cells were seeded at 1×10^5 cells per 35 mm dish and the following day were transfected with 8 μg/ml of DMRIE-C (Invitrogen) plus 1 μg of plasmid CMVβgal mixed with 2 μg of pXP2 in serum-free medium. Twenty-four hours after transfection, the cultures were replenished with complete medium and placed in hypoxic or normoxic conditions for the designated time periods. Thereafter, the cells were harvested and the enzymatic activity determined. Luciferase and β-galactosidase activities were measured on a TD-20/20 Luminometer (Promega, WI) and a colorimetric plate reader (Labsystems), respectively, using kits from Promega (UK).

Cell cycle analysis

Cell cycle distribution was evaluated by propidium iodide staining of nuclei and flow cytometric analysis (Li et al., 1997). 1×10^6 cells were fixed with 2 ml cold 70% (v/v) ethanol in PBS, immediately mixed and slowly agitated for 15 minutes at room temperature. After two washes with PBS, 0.7 ml of 0.2 mg/ml pepsin (Sigma) in 2 M HCl was added to the pelleted cells for simultaneous proteolysis and DNA denaturation at 37°C for 30 minutes. The hydrolysis was terminated by addition of 2 ml of 1 M Tris (pH 10). Cells were washed twice and incubated in 0.3 ml PBS containing 10 μg/ml propidium iodide for 15 minutes on ice. To determine the proportion of cells in various phases of the cell cycle, propidium iodide staining of the DNA in 2×10^4 nuclei was quantified using a Becton Dickinson FACScan flow cytometer.

Analysis of apoptosis

The terminal deoxynucleotidyltransferase-mediated dUTP end

labelling (TUNEL) assay was used to identify cell apoptosis. Cells were seeded onto chamber slides (Nunc) at a density of 2×10^4 cells per 0.5 ml per well, grown overnight and subjected to hypoxic or normoxic conditions. Fragmented DNA staining of apoptotic cells was carried out using a commercial kit (Roche, Mannheim, Germany). Briefly, the cells were rinsed twice with pre-warmed PBS followed by fixation using 4% paraformaldehyde for 1 hour at room temperature. The fixed cells were permeabilised by incubation with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes. The cells were rinsed again with PBS and incubated with 50 μl per sample of TUNEL reaction mixture for 1 hour at 37°C. A negative control was included in each staining wherein only the labelling solution was added.

TUNEL staining of cells for FACS analysis was carried out using the same kit as described above. The cells were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 1 hour at room temperature. After one wash with PBS, cells were permeabilised using 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice, followed by two washes with PBS. The permeabilised cells were stained with 50 μl/sample of the TUNEL reaction mixture containing the enzyme or the labelling solution without the enzyme as a negative control and incubated for 1 hour at 37°C in a humidified chamber in the dark. Finally, the cells were washed twice with PBS and resuspended in 0.5 ml PBS for FACS analysis. For quantification of anti- and pro-apoptotic proteins, cells were harvested, fixed and permeabilised as described above. Antibodies to Bcl-2, Bcl-X_L, Bax, Mcl-1, caspase-3 and caspase-8 (BD Biosciences) were incubated with 5×10^5 cells/tube for 1 hour on ice. Pre-immunised rabbit serum or isotype matched mouse immunoglobulins were used as negative controls. The FITC-conjugated secondary antibodies (DAKO) were added and incubated for 30 minutes on ice. Flow cytometric analysis was carried out on a FACScan.

Results

Hypoxia upregulates CD105 protein levels

HDMECs collected from normoxic or hypoxic cultures were stained with mAb E9, which reacts specifically with human CD105 (Pichuantes et al., 1997), and cell surface CD105 expression was quantified by flow cytometry. CD105 levels were markedly elevated under hypoxic conditions compared with normoxic conditions, especially after 16 hours (Fig. 1A). The geometric mean fluorescence intensity increased from 149.5±13.5 in normoxic conditions to 278.0±19.0 after 16 hours of hypoxic culture, that is, an 86.3% increase in cell surface CD105. FACS analysis was used to compare the reactivity of mAbs E9 and 44G4 against human CD105 on the same HDMECs (Gougos and Letarte, 1988; Pichuantes et al., 1997). Both mAbs reacted with ~100% of the confluent cells in normoxic conditions. mAbs 44G4 and E9 bound equally well to HDMECs, resulting in almost identical results under hypoxic conditions (data not shown). Next, analysis of total CD105 present in cellular extracts was carried out by immunoblotting analysis. Cell extracts were prepared from the same batch of HDMECs used for flow cytometry analysis and subjected to electrophoresis under non-reducing conditions, blotted to PVDF membrane and probed using a mAb against CD105 and a goat anti-α-actin antibody. In contrast to flow cytometry analysis, which detects only membrane-bound antigen, immunoblotting identifies total CD105. As depicted in Fig. 1B, two specific bands with molecular weights of approximately 180 and 95 kDa were observed. The 180 kDa band corresponds to the mature dimeric form of CD105 and showed a ~threefold greater intensity after 16 hours of hypoxic culture compared with normoxic controls. The smaller band is

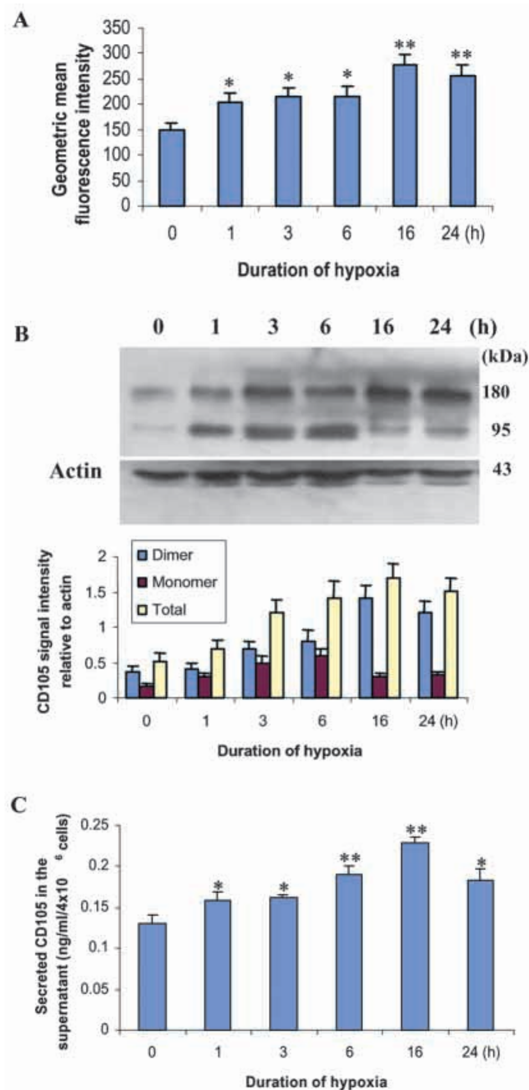

Fig. 1. Hypoxia induces CD105 protein expression. HDMECs were cultured under hypoxic conditions for up to 24 hours, and CD105 expression was quantified by flow cytometry (A), immunoblotting (B) and ELISA (C) as described in Materials and Methods. (A) Hypoxic culture resulted in a maximal cell surface expression of CD105 at 16 hours (86.3% increase). The data represent five samples at each time point collected from five separate experiments (*P<0.05 and **P<0.01 compared with 0 hours as analysed by one-way ANOVA followed by the Duncan test). (B) Cell extracts were resolved on 4-7.5% SDS-PAGE under non-reducing conditions and electrophoretically transferred onto membranes. The blot was probed using mAb E9 and a rabbit anti-α-actin antibody. The maximal expression (as quantified on a densitometer) of CD105 was seen at 16 hours of hypoxic culture. Similar results were observed when the blots were reprobed using mAb 44G4 (data not shown). The bar chart shows the CD105 signal intensity relative to actin, pooled from three experiments. (C) Conditioned medium was collected, and soluble CD105 levels were quantified using a chemiluminescence ELISA system. CD105 levels peaked at 16 hours of hypoxic culture. The data represent six replicates at each time point pooled from three separate experiments (*P<0.05 and **P<0.01 compared with 0 hours as analyzed by one-way ANOVA followed by the Duncan test). Vertical bars indicate the standard error of the means.

likely to be a monomer of CD105 (Paquet et al., 2001). Both the dimer and the monomer forms of CD105 were markedly increased under hypoxic conditions and were maximal at 16 hours, indicating that hypoxia significantly elevated the levels of total cellular CD105 protein. When the blots were stripped and reprobed using mAb 44G4, the same bands were observed, with similar intensity, confirming that the two mAbs to human CD105 had almost equal reactivity.

CD105 levels in the blood of patients with breast cancer and those with atherosclerosis are elevated and associated with disease progression. Thus CD105 could be a marker for certain clinical conditions (Blann et al., 1996; Li et al., 2000c). In the present study, CD105 levels were quantified in conditioned medium collected from cells used for protein and mRNA analysis. The levels of secreted CD105 were increased after 1 hour of oxygen deprivation, peaked at 16 hours in hypoxic cultures and remained higher than controls at 24 hours (Fig. 1C). However the biological implications of the increase in CD105 are unknown. The profile of secreted CD105 in the oxygen-deprived medium was comparable to cell surface CD105 observed by flow cytometry and total CD105 estimated by immunoblotting analysis.

Hypoxia upregulates CD105 mRNA levels and promoter activity

To analyse whether the levels of CD105 transcripts were also affected by hypoxia, total RNA was fractionated and hybridised with radiolabelled CD105 and GAPDH cDNAs, visualised on a phosphorimager and quantified using a densitometer. Hypoxia elevated CD105 mRNA levels, which was evident even after 1 hour, compared with normoxic culture (Fig. 2A). The highest expression of CD105 mRNA (a ~threefold increase) occurred at 3 hours. The relatively weak signal for CD105 mRNA after 24 hours of hypoxic culture was considered to reflect hypoxia-induced cell cycle arrest and apoptosis, which was confirmed by propidium iodide staining and a TUNEL assay, as described below. These low levels of CD105 mRNA were concomitant with high expression levels of CD105 protein (Fig. 1), in agreement with the high stability of the protein (Paquet et al., 2001). To assess whether the increased CD105 expression in hypoxic conditions was due to an increased promoter activity, CD105 promoter activity was examined using a luciferase reporter assay. CD105 promoter activity was significantly induced by hypoxia, reaching a maximal level after 3-6 hours of hypoxic culture (Fig. 2B). Longer durations of hypoxia (more than 16 hours) led to basal levels of the promoter activity, probably owing to cell cycle arrest and apoptosis.

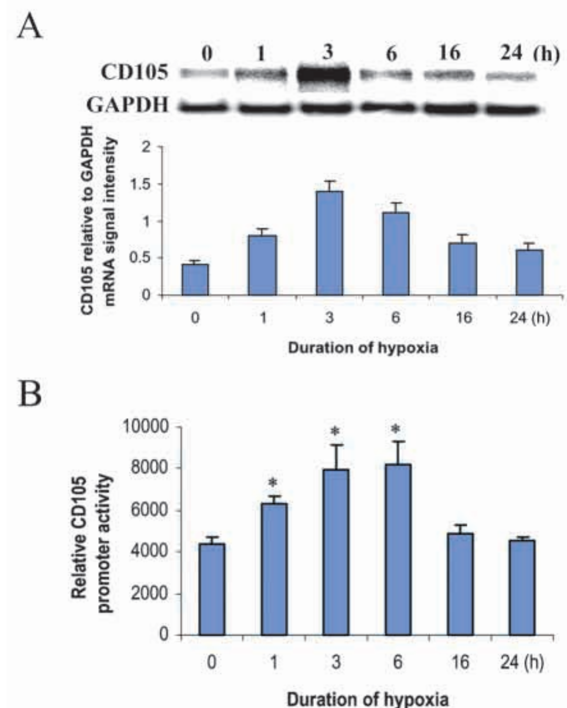

Fig. 2. Effect of hypoxia on CD105 transcription. (A) Northern blot analysis. Total RNA was extracted from HDMECs and fractionated on a 1% denaturing agarose gel. After blotting onto a nitrocellulose membrane, the fractionated RNA was probed using ^{32}P-labelled cDNAs for CD105 or GAPDH, visualised on a phosphorimager and quantified using a densitometer. Maximal expression of CD105 mRNA was observed at 3 hours with a ~threefold increase over normoxic culture. The bar chart represents CD105 mRNA relative to GAPDH mRNA signal intensity collected from three experiments. (B) Hypoxia activates the CD105 promoter. HDMECs (the same batch of cells as for panel A) co-transfected with plasmid pXP2/pCD105/luc and CMVβgal were grown under hypoxic condition for up to 24 hours. Luciferase activity was determined and normalised to β-galactosidase activity. CD105 promoter activity peaked between 3 hours and 6 hours of culture (*$P<0.05$ and **$P<0.01$ compared with 0 hours as analysed by one-way ANOVA followed by the Duncan test). Data represent six replicates at each time point collected from three separate experiments. Vertical bars indicate standard error of the means.

Hypoxia induces cell cycle arrest and apoptosis in HDMECs

Since persistent hypoxia induces alterations in cell cycle and apoptosis in certain cell types (Carmeliet et al., 1998), an analysis of cell cycle and apoptosis was performed in hypoxic HDMECs. Prolonged exposure to hypoxia (24 hours) led to a dramatic alteration in cell cycle, that is, more cells arrested at the G0/G1 phases and fewer cells undergoing DNA synthesis (S phase). Moreover, DNA fragmentation was observed in a considerable proportion of cells (approximately 21% of the total population – blue profile) after 24 hours of hypoxic culture, implying massive cell apoptosis (Fig. 3A). A TUNEL assay was carried out in parallel on the same batch of cells to identify cells with fragmented DNA. Under normoxic conditions, few apoptotic cells were observed. In contrast, in cells grown under hypoxic conditions DNA fragmentation occurred in a time-dependent manner: prolonged exposure to oxygen deprivation induced substantial cell apoptosis (Fig. 3B). The highest proportion of apoptotic cells was detected after 24 hours of hypoxic culture, which is consistent with the data obtained by propidium iodide staining.

Hypoxia alters expression of pro- and anti-apoptotic markers

To investigate the mechanisms by which hypoxia induces cell apoptosis, the protein expression of the known anti-apoptotic

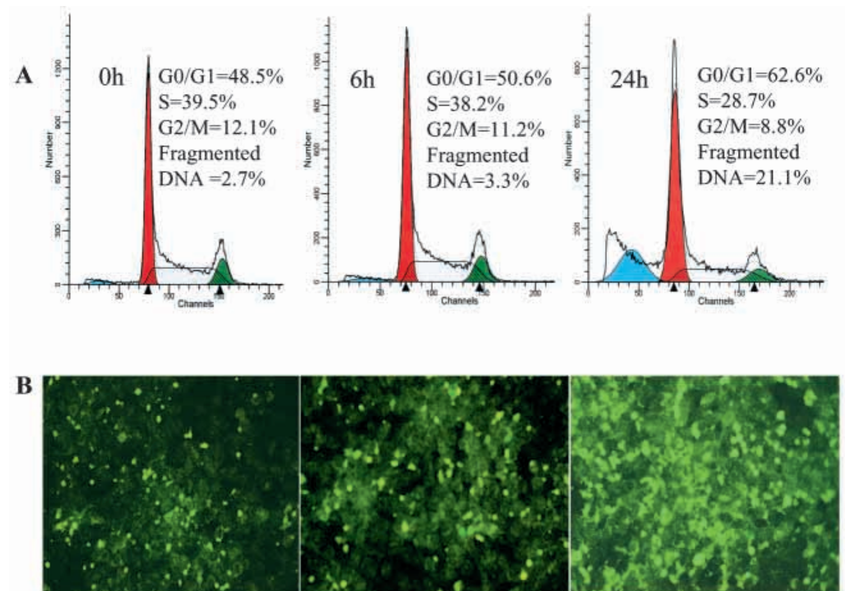

Fig. 3. Cell cycle analysis and TUNEL staining of cells cultured under hypoxic conditions. HDMECs were cultured under hypoxic conditions for 0, 6 or 24 hours as indicated. (A) For cell cycle analysis, DNA was stained using propidium iodide and analysed by flow cytometry. Hypoxic culture resulted in an increased number of cells arrested at the G0/G1 phases and an increased level of DNA fragmentation (blue profile). (B) TUNEL staining was performed on the same batch of cells used for cell cycle analysis. Apple green staining of nuclei corresponds to apoptotic cells. Hypoxic culture led to an increased number of apoptotic cells (original magnification: ×250). All experiments were performed at least three times and produced similar results.

markers Bcl-2, Bcl-X_L, Mcl-1 and the pro-apoptotic markers Bax, caspase-3 and caspase-8 were quantified in cells under normoxia or hypoxia for 24 hours. As shown in Fig. 4, Bcl-X_L and Mcl-1 were significantly suppressed, Bax and Caspase-3 were markedly raised and caspase-8 slightly raised in hypoxia. Although Bcl-2 was elevated in hypoxia, the ratio of Bcl-2 to Bax was lowered from 1.36 in normoxia to 0.79 in hypoxia. These data suggest that hypoxia induces cell apoptosis through the downregulation of Bcl-X_L and Mcl-1, upregulation of Bax, caspase-3 and caspase-8 and the lowering of the Bcl-2 to Bax ratio.

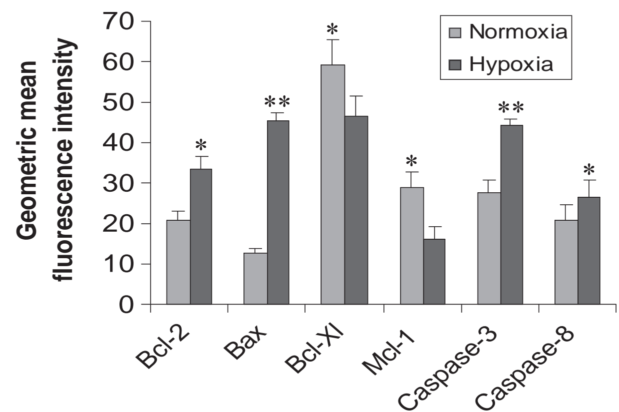

Fig. 4. Analysis of anti- and pro-apoptotic proteins under hypoxic conditions. Cells collected after 24 hours of culture under normoxic or hypoxic conditions were subjected to immunostaining using specific antibodies and FACS analysis. The ratio of Bcl-2 to Bax dropped from 1.36 in normoxia to 0.79 in hypoxia. Data were expressed as means±s.e.m. pooled from duplicate samples of three experiments (*P<0.05 and **P<0.01, student's t-test).

Suppression of CD105 gene expression using an antisense approach

To investigate the role of CD105 in HDMECs, CD105 gene expression was specifically inhibited using an antisense approach. There have been numerous reports in the literature using the same experimental approach, and the method of transfection is mild and well-tolerated. Thus, examining the effect of hypoxia on the transfected cells is considered to be appropriate. In comparison with HUVECs, in which CD105 was significantly reduced using 0.25 nM/ml of AS ODN plus 5.6 μg/ml of lipofectACE (Invitrogen) (Li et al., 2000a), HDMECs were less responsive to such a formulation. However, the efficacy was improved by using an increased concentration of AS ODN (0.5 nM/ml) plus 8 μg/ml of DMRIE-C (Invitrogen). FACS and immunoblotting analysis revealed a 55-60% reduction in CD105 protein levels in the AS-ODN-treated cells after 72 hours of incubation in normoxia and 24 hours exposure to hypoxia, compared with SC-ODN-treated (0.5 nM/ml plus 8 μg/ml DMRIE-C) or untreated cells under the same conditions (Fig. 5A,B). To investigate whether CD105 mRNA was altered by ODN treatment, northern blotting was carried out. As depicted in Fig. 5C, CD105 mRNA was decreased by AS but not by the SC ODN, demonstrating that specific degradation of CD105 mRNA was one of the mechanisms involved in the antisense effect. The specificity of the antisense effect was further verified by quantifying the expression of CD31, vWF, TGFβ receptor I and receptor II in the untreated HDMECs or HDMECs treated with either AS or SC ODN. As determined by FACS and immunoblotting, these cell membrane proteins were not affected by the ODN treatment, whereas CD105 protein was significantly reduced by the AS ODN (data not shown). The selective inhibition of CD105 gene expression by the AS ODN can also be seen in Fig. 5, where CD105 but not CD31 protein (A), CD105 but not GAPDH mRNA (C) were markedly decreased by the AS ODN.

Inhibition of CD105 gene expression heightens hypoxia-induced cell apoptosis

Hypoxia stimulates angiogenesis by upregulating expression of angiogenic factors but causes apoptosis as well. A balance between the two forces determines the fate of the cell. To

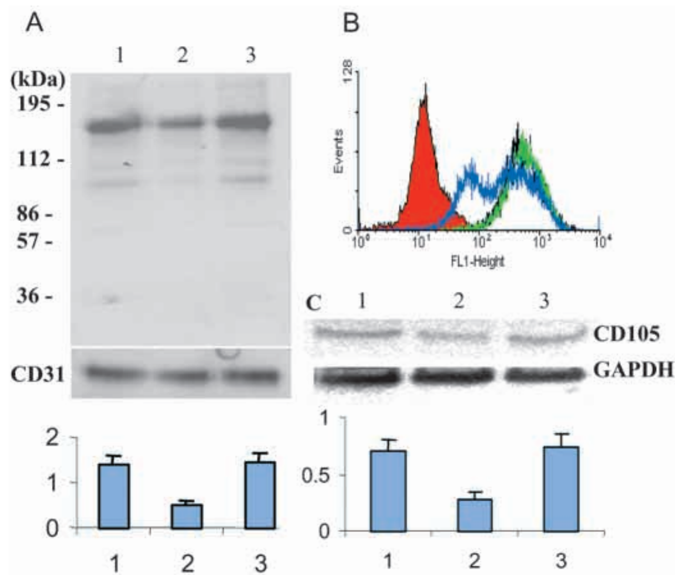

Fig. 5. CD105 mRNA and protein expression in ODN-treated and untreated cells. HDMECs were cultured for 72 hours in normoxia in the presence or absence of ODN plus DMRIE-C, followed by exposure to hypoxia for 24 hours. CD105 protein levels were assessed by immunoblotting and FACS, and mRNA levels were assessed by northern blotting. (A) Immunoblotting revealed that CD105 protein was reduced by ~58% in AS-ODN-treated cells (lane 2) compared with SC-ODN-treated (lane 3) and untreated cells (lane 1). The bar chart indicates the ratio of CD105 relative to CD31 signal intensity collected from three experiments (means±s.e.m.). (B) FACS analysis of cell surface CD105 indicated a ~60% reduction in AS-ODN-treated (blue profile) compared with SC-ODN-treated (green profile) or untreated (black profile) cells. The red profile represents a negative control, where mAb E9 was substituted with pre-immunised mouse serum. The plot is a representative of five similar experiments. (C) Northern blotting of CD105 and GAPDH mRNA showed that CD105 mRNA was markedly degraded by AS ODN (lane 2) but not by SC ODN (lane 3). Lane 1 is mRNA extracted from untreated cells, and it has no evident alteration compared with lane 3. The bar chart shows CD105 mRNA, relative to GAPDH mRNA signal intensity, pooled from three experiments (means±s.e.m.).

elucidate whether upregulation of CD105 in HDMECs under hypoxic stress is of functional importance, we quantified cell apoptosis of the AS-ODN-treated, SC-ODN-treated or untreated HDMECs. Exposure of untreated cells to hypoxic stress for 24 hours elicited a significant cell apoptosis, 48.5% under hypoxia in contrast to 4.3% under normoxia. Hypoxia induced a maximal cell apoptosis in the CD105-depressed out of the three groups of cells cultured in complete growth medium without exogenously added TGFβ1 or TGFβ3. Thus, the geometric mean fluorescence intensity was 139.1±13.3 in untreated, 150.7±9.0 in SC-ODN-treated and 193.1±10.6 in AS-ODN-treated cells ($P<0.05$, AS-ODN-treated, compared with either SC-ODN-treated or untreated cells) (Fig. 6A). The percentage of apoptotic cells correlates well with the geometric mean fluorescence intensity, which was 66.5% in CD105-depressed compared with 48.5% in untreated or 48.2% in SC-ODN-treated cells ($P<0.05$) (Fig. 6B). Administration of the neutralising antibodies to TGFβ1 and TGFβ3 produced no significant alteration either in the percentage of apoptotic cells or in their fluorescence intensity, demonstrating that CD105 is able to act independently of TGFβ in preventing cell apoptosis under hypoxic stress.

Hypoxia and TGFβ1 synergistically augment cell apoptosis in CD105-depressed cells

CD105 regulate TGFβ1 signalling in endothelial cells and in CD105 transfectants (Lastres et al., 1996; Letamendia et al., 1998; Li et al., 2000a) but how CD105 and TGFβ1 interact under hypoxic conditions to control cell apoptosis has not been examined. Therefore we investigated this issue using the AS-ODN-treated (CD105-deficient) cells, the SC-ODN-treated and untreated cells. As shown in Fig. 6, TGFβ1 induced a marginal increase in apoptotic cells in the SC-ODN-treated and the untreated cells. In contrast, it caused a considerable increase in apoptotic cells in the CD105-depressed cells. The fluorescence intensity of CD105-deficient cells was 193.1±10.6 in the absence of TGFβ1, 229.7±10.7 at 0.1 ng/ml of TGFβ1, and 298.2±11.5 at 10 ng/ml of TGFβ1, in contrast to 150.7±9.0, 157.9±8.7 and 175.6±10.8, respectively, in SC-ODN-treated cells ($P<0.05$ or $P<0.01$, significant difference between the two groups at each same concentration of TGFβ1) (Fig. 6A). This represents 28.1%, 45.5% and 69.8% increases in fluorescence intensity in the CD105-deficient cells compared with SC-ODN-treated cells at the same concentrations of TGFβ1. A higher proportion of apoptotic cells was observed in the CD105-deficient cells compared with either the SC-ODN-treated or untreated cells at the same concentration of TGFβ1 (Fig. 6B). The percentage of apoptotic cells increased from 48.2±2.6, 51.3±3.0 and 56.9±3.0 in SC-ODN-treated cells at 0, 0.1 and 10 ng/ml of TGFβ1 to 66.5±2.3, 75.7±2.6 and 87.4±2.5 in the AS-ODN-treated cells at the same concentrations of TGFβ1 ($P<0.05$ or $P<0.01$, significant difference between the two groups at each same concentration of TGFβ1). These data demonstrate that CD105 functions as an anti-apoptotic protein in the presence of TGFβ1 under hypoxic stress.

Discussion

Hypoxia is a major stimulus of neovascularisation under many conditions, including tumour angiogenesis, collateral vessel formation in ischaemic cardiovascular diseases and proliferative retinopathy (Semenza, 2000; Marti and Risau, 1999; Richard et al., 1999; Smith et al., 1997). CD105 is upregulated in all these disease states, although its role in angiogenesis is not fully understood. In this study we demonstrate that hypoxia activates the CD105 gene promoter, augmenting its mRNA transcription and protein translation. Most importantly, our data reveal that CD105 exerts an anti-apoptotic effect in endothelial cells under hypoxic stress, either in the absence or in the presence of TGFβ1. In view of these observations, it can be speculated that hypoxia-initiated neovascularisation is mediated, at least in part, through the stimulation of CD105 expression in ECs.

The notion that CD105 is associated with angiogenesis initially came from the observation that a mAb to CD105 reacts most strongly with the endothelium of tumours but only weakly or not at all with normal tissues (Wang et al., 1993). Subsequently, numerous studies using mAbs to CD105 on a broad range of tissues have provided supportive evidence that

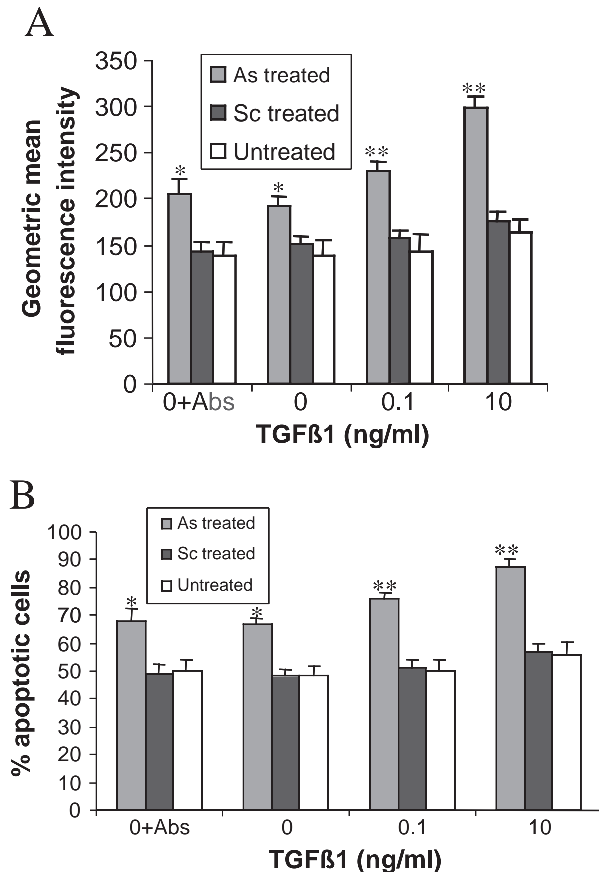

Fig. 6. Effect of CD105 antisense ODN on cell apoptosis. Three groups of cells, AS- or SC-ODN-treated or untreated HDMECs, were exposed to hypoxia and/or TGF-β1 for 24 hours, TUNEL-stained and analysed by FACS. The fluorescence intensity (A) and percentage of apoptotic cells (B) were determined. The apoptotic effect of TGFβ1 was observed to be concentration dependent, with the maximal effect of TGFβ1 at 10 ng/ml. Data were expressed as means±s.e.m. collected from six samples of three experiments. (**P*<0.05 and ***P*<0.01, one-way ANOVA followed by the Duncan test).

CD105 is indeed upregulated in many types of tissues undergoing angiogenesis (Kumar et al., 1996; Miller et al., 1999; Seon and Kumar, 2001; Wikstrom et al., 2002). The expression of CD105 in blood vessels of breast and lung cancer tissues was found to be correlated with poor prognosis (Kumar et al., 1999; Tanaka et al., 2001), which suggests that CD105 promotes tumour progression. A conclusive demonstration of the crucial role of CD105 in vascular development came from CD105-knockout mice, which had severe defects in angiogenesis; the homozygotes died in utero owing to impaired development of vasculature (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). In line with these observations, CD105 expression correlates with activation/proliferation of tumour endothelial cells (Kumar et al., 1999; Miller et al., 1999). These findings provide compelling evidence that CD105 is important for angiogenesis. However, the underlying mechanism of how CD105 promotes angiogenesis is not clear. Existing data support the view that CD105 overexpression in transfectants weakens the effects of TGFβ1 and that these effects might be cell type dependent (Lastres et al., 1996; Letamendia et al., 1998). In addition, suppression of CD105 in ECs with antisense ODN in combination with TGFβ1 led to a strong inhibition of angiogenesis (Li et al., 2000a). These observations indicate that CD105 modulates TGFβ1 signalling in these cells and that an adequate level of CD105 in ECs is required for angiogenesis.

The expression of CD105 is likely to be regulated by factors involved in angiogenesis and/or vessel remodelling. Here we have demonstrated that CD105 is markedly induced in ECs by exposure to hypoxia. CD105 promoter activity and mRNA transcription responded rapidly to hypoxia, which suggests that it is regulated at the transcriptional level. In this regard, a consensus hypoxia responsive element (HRE) has been recently characterized within the CD105 promoter (Sanchez-Elsner et al., 2002). The CD105 protein was maintained at high levels until the end of the experiment. The half-life of CD105 on the cell surface has been estimated to be 17 hours, measured by metabolic labelling (Paquet et al., 2001). Thus, once it is upregulated, CD105 can remain in the EC, exhibiting a prolonged effect. The relatively low promoter activity and mRNA level after 24 hours of hypoxic culture is apparently a consequence of hypoxia-induced cell cycle arrest and apoptosis. This is in line with previous observations that CD105 is more strongly expressed in activated/mitotic cells than quiescent cells, suggesting that it is a proliferation-associated gene. These findings also highlight the potential value of the CD105 promoter for gene therapy in cancer and ischaemic diseases (Brekken et al., 2002; Velasco et al., 2001). Genes driven by the CD105 promoter may be more strongly and specifically expressed in the oxygen-deprived tissues and exert therapeutic effects.

Hypoxia is the primary driving force for neovascularisation. The major angiogenic factors, such as VEGF and bFGF, are upregulated by hypoxia, and promote angiogenesis and thus improves oxygen supply to the hypoxic tissues (Mukhopadhyay et al., 1995; Sakaki et al., 1995; Schweiki et al., 1992). On the other hand, persistent hypoxic stress induces EC apoptosis (Carmeliet et al., 1998; Hogg et al., 1999). In this study we have shown that the pro-apoptotic proteins Bax, caspase-3 and caspase-8 were elevated and that the anti-apoptotic proteins Bcl-X_L and Mcl-1 were significantly decreased under hypoxic stress. Although anti-apoptotic Bcl-2 was upregulated, the ratio of Bcl-2 to Bax was considerably lowered under hypoxic stress compared with normoxia. Therefore, the mechanisms of hypoxia-induced EC apoptosis largely depend on the lowered Bcl-2 to Bax ratio, Bcl-X_L and Mcl-1 and increased expression of caspase-3 and caspase-8. These findings are in agreement with previous reports (Khurana et al., 2002; Taraseviciene-Stewart et al., 2001; Wang et al., 2002).

Because CD105 is strongly expressed in the HDMECs under hypoxic conditions, we adopted an antisense approach to suppress CD105 expression so that its function could be specifically addressed. The CD105 protein and mRNA levels were considerably reduced by the antisense ODN but not affected by the control SC ODN. Using the CD105-deficient cells and the control cells, an important function of CD105 has been discovered, which is that it acts as an anti-apoptotic protein in ECs under hypoxic stress. Such an effect was observed in the absence of TGFβ1 and TGFβ3, indicating that

CD105 functions beyond its role as a receptor for TGFβ1 and TGFβ3. In fact, only approximately 1% of membrane-bound CD105 binds to TGFβ1 and TGFβ3 (Cheifetz et al., 1992). The function of the majority of CD105 that does not bind to TGFβ remains unclear. Our data suggest that the non-TGFβ-binding CD105 in EC plays a self-protective role against apoptotic factors such as hypoxia. The protective role of CD105 against apoptosis has been further confirmed using a completely different system – murine endothelial cells from CD105 heterozygous knockout mice (J.M.L.-N., C.L., S.K. et al., unpublished). The addition of TGFβ1 to control cells under hypoxia induced a marginal increase in the proportion of apoptotic cells, whereas the apoptotic action of TGFβ1 was considerably increased in the CD105-depressed cells, demonstrating that the upregulation of CD105 protects ECs from the apoptotic action of TGFβ1. In an in vivo environment, where both hypoxia and TGFβ1 can co-exist, the augmented expression of CD105 may act as an anti-apoptotic force, so as to protect ECs against hypoxia and against TGFβ1-induced apoptosis.

In conclusion, we have demonstrated that hypoxia activates the CD105 promoter and significantly induces its gene expression in human microvascular ECs. The upregulated CD105 exhibits a role in self-protection against hypoxia and TGFβ1-induced cell apoptosis, resulting in an enhanced survival ability of EC under hypoxic stress. These findings may lead us to a better understanding of the functions of CD105 in angiogenesis and of the pathogenesis of other CD105-related vascular disorders.

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