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RESEARCH PAPER

Sustained contraction and loss of NO production in TGFβ_1-treated endothelial cells

M Watanabe[1,2], M Oike[1], Y Ohta[1], H Nawata[2] and Y Ito[1]

[1]*Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and* [2]*Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Background and purpose: Transforming growth factor β_1 (TGFβ_1) is generated in atherosclerotic and injured vessel walls. We examined whether the endothelial-to-mesenchymal transdifferentiation induced by TGFβ_1 affects endothelial functions.
Experimental approach: Bovine aortic endothelial cells (BAECs) were treated with 3 ng ml^{-1} TGFβ_1 for 7 days. Contraction of TGFβ_1-treated BAECs was assessed by collagen gel contraction assay. Protein expression and phosphorylation were assessed by Western blotting. Intracellular Ca^{2+} concentration and NO production were measured using fura2 and DAF-2, respectively.
Key results: TGFβ_1-treated BAECs showed dense actin fibers and expressed smooth muscle marker proteins; they also changed into smooth muscle-like, spindle-shaped cells in collagen gel cultures. ATP (10 μM) induced a gradual contraction of collagen gels containing TGFβ_1-treated BAECs but not of gels containing control BAECs. ATP-induced contraction of TGFβ_1-treated BAECs was not reversed by the removal of ATP but was partially suppressed by a high concentration of sodium nitroprusside (1 μM). TGFβ_1-treated BAECs showed sustained phosphorylation of myosin light chain in response to ATP and low levels of basal MYPT1 expression. ATP-induced Ca^{2+} transients as well as eNOS protein expression were not affected by TGFβ_1 in BAECs. However, ATP-induced NO production was significantly reduced in TGFβ_1-treated BAECs. Anti-TGFβ_1 antibody abolished all of these TGFβ_1-induced changes in BAECs.
Conclusions and Implications: Mesenchymal transdifferentiation induced by TGFβ_1 leads to sustained contraction and reduced NO production in endothelial cells. Such effects, therefore, would not be beneficial for vascular integrity.
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Keywords: TGFβ_1; endothelium; smooth muscle; contraction; nitric oxide

Abbreviations: α-SM actin, α-smooth muscle actin; BAECs, bovine aortic endothelial cells; BASMCs, bovine aortic smooth muscle cells; DAF-2, diaminofluorescein-2; DMEM, Dulbecco's Modified Eagle's Medium; ET-1, endothelin-1; MLC, myosin light chain; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1; p-MLC, phosphorylated myosin light chain; p-MYPT1, phosphorylated MYPT1; SNP, sodium nitroprusside; TGFβ_1, transforming growth factor β_1

Introduction

Transforming growth factor β_1 (TGFβ_1) is a multifunctional cytokine involved in the regulation of cell proliferation, differentiation and survival in many cell types (Roberts and Sporn, 1993). TGFβ_1 is secreted from various cell types in the vascular wall such as smooth muscle cells (Yue *et al*., 1994), endothelial cells (Hannan *et al*., 1988), platelets (Ross *et al*., 1986) and macrophages (Assoian *et al*., 1987), especially in injured vessels, atherosclerotic lesions and post-angioplasty restenotic lesions (McCaffrey, 2000; Grainger, 2004). Vascular endothelial cells are one of the main targets of TGFβ_1 (Frater-Schroder *et al*., 1986; Pollman *et al*., 1999), and it is well-established that TGFβ_1 inhibits endothelial proliferation (Frater-Schroder *et al*., 1986; Heimark *et al*., 1986) and migration (Krizbai *et al*., 2000). It has already been proposed that these effects of TGFβ_1 on endothelium would lead to the slowing of re-endothelialization of the injured vascular surface and allow time for recruitment of smooth muscle cells into the site of injury (Heimark *et al*., 1986).

Another distinct characteristic of TGFβ_1 is the induction of mesenchymal transdifferentiation and morphological changes in endothelial cells (Sutton *et al*., 1991; Arciniegas *et al*., 1992). TGFβ_1 has been reported to induce the expression of smooth muscle markers such as α-smooth muscle actin (α-SM actin), SM22α, calponin and SM myosin in aortic and pulmonary artery endothelial cells (Arciniegas *et al*., 1992; Frid *et al*., 2002). These authors speculated that the transformed endothelial cells may serve as a source of contractile smooth muscle cells in the repair of

Correspondence: Dr M Oike, Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan.
E-mail: moike@pharmaco.med.kyushu-u.ac.jp
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atherosclerotic lesions and/or injured vascular walls (Arciniegas *et al*., 1992; Frid *et al*., 2002). TGFβ_1-induced endothelial–mesenchymal transdifferentiation was also reported in human and ovine valvular endothelial cells, in which cell migration was markedly increased, thereby suggesting that TGFβ_1 may play a significant role in replenishing the interstitial cells of cardiac valves (Paranya *et al*., 2001). However, none of these previous studies have examined the contractility of TGFβ_1-treated endothelial cells.

It is also still controversial whether TGFβ_1 is beneficial for endothelial NO production or not. Earlier studies showed that TGFβ_1 increased NO production via upregulation of eNOS protein expression, thereby suggesting a vasoprotective role of TGFβ_1 in injured vessels (Poppa *et al*., 1998; Tai *et al*., 2004). In contrast, a recent report has shown that the binding of TGFβ_1 to its receptor expels eNOS from caveolae and reduces its activity in human endothelium (Schwartz *et al*., 2005). As mechanical stress-induced change in cell shape is quite important for endothelial NO production (Kimura *et al*., 2000), it would be also possible that TGFβ_1-induced morphological change may affect NO production in endothelial cells.

We have focused in this study on the functional significance of TGFβ_1-induced endothelial–mesenchymal transdifferentiation. For this purpose, we examined the effects of TGFβ_1 on contractility and NO production, as representative features of smooth muscle and endothelial functions, respectively, in bovine aortic endothelial cells (BAECs). Our results revealed, for the first time, that TGFβ_1 changes endothelial cells into non-smooth muscle contractile cells.

Materials and methods

Cell culture

Thoracic aortas of 1-year-old calves were obtained from the local slaughterhouse, and BAECs and aortic smooth muscle cells (BASMCs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum as previously described (Kimura *et al*., 2001a, 2002).

Treatments with TGFβ_1 and ani-TGFβ_1 antibody

Treatment with TGFβ_1 consisted of incubating the non-confluent cells with culture medium containing TGFβ_1 for various periods. Effects of neutralizing antibody for TGFβ_1 were examined by using culture medium that was pre-incubated with 3 ng ml^{-1} TGFβ_1 and 300 ng ml^{-1} anti-TGFβ_1 antibody for 1 h at room temperature.

Immunological staining of fibrous actin

Fibrous actin was stained using rhodamine-conjugated phalloidin according to the previously reported method (Knudsen and Frangos, 1997).

Gel contraction assay

Contractility of cultured BAECs and BASMCs were examined with the gel contraction assay (Kimura *et al*., 2002). The cells were cultured on plates for 7 days with or without TGFβ_1, and were harvested by trypsinization. The harvested cells were re-suspended in DMEM containing 0.2% collagen type IA at a density of 4×10^5 cells ml^{-1}, and 0.5 ml of the cell suspension per well was poured into a 24-well culture plate. The plate was kept at 37°C for 10 min to form a gel, and 1 ml of culture medium per well was added to the gel (see diagrams in Figure 3a). The gels were cultured for 3 days and used for the contraction assay. The lateral surface of the gel was carefully detached from the culture well with a fine needle. The culture plate was then placed on a hotplate (MP-10DM; Kitazato Supply, Shizuoka, Japan) and kept at 37°C. The gel surface images were captured with a digital camera (QV-800SX, Casio, Tokyo, Japan) every 1 min throughout the experiment. Contraction of the gel was then evaluated by measuring its surface area with an image analysis software (Adobe Photoshop, Adobe Systems Inc., San Jose, CA, USA).

Western blot analysis

Expressions of smooth muscle marker proteins, MLC, MYPT1 and eNOS protein, and the amount of p-MLC and p-MYPT1 were assessed with chemiluminescence Western blotting. Cell lysates were prepared after each pretreatment and separated by electrophoresis. Western blot analysis was carried out by using the relevant antibody.

In each experiment, the bands were detected with chemiluminescence system (SuperSignal West Dura, Pierce Co., Rockford, IL, USA) and analysed with a lumino image analyzer (FAS-1000, Toyobo, Osaka, Japan).

Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured in non-confluent BAECs with Fura-2 by using an Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD, USA), as previously described (Oike *et al*., 2000). For the statistical analysis of $[Ca^{2+}]_i$, results from 20 to 30 cells in a coverslip were averaged and treated as one data point.

Measurement of intracellular production of NO

To determine the intracellular NO production in BAECs, an NO-sensitive fluorescent dye diaminofluorescein-2 (DAF-2) (Kojima *et al*., 1998) was used. Non-confluent cells grown on coverslip were incubated with a diacetylated form of DAF-2 (DAF-2/DA, 10 μM) for 20 min at 37°C. DAF-2 fluorescence was measured and analysed as previously described (Kimura *et al*., 2001b).

Solutions

Krebs solution used in Ca^{2+} and NO measurements contained (mM); NaCl 132.4, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 11.5, HEPES 11.5 and pH was adjusted to 7.4 by NaOH.

Statistics

Pooled data were expressed as mean ± s.e.m. values. Statistical significance was assessed with Student's unpaired *t*-test. Values of $P<0.05$ were considered to show significant differences between means.

Materials

Anti-TGFβ_1, anti-troponin (clone hCP), anti-SM myosin (clone HSM-V), anti-myosin light chain (MLC, clone MY-21) and anti β-actin (clone AC-15) antibodies were purchased from Sigma (St Louis, MO, USA). Anti-myosin phosphatase target subunit 1 (MYPT1) and anti-phosphorylated MYPT1 (p-MYPT1, Thr 696) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-SM actin (clone 1A4) was purchased from Dako (Glostrup, Denmark). Anti-eNOS antibody was purchased from StressGen Biotechnologies (San Diego, CA, USA). Anti-phosphorylated MLC (p-MLC) antibody (Thr18/Ser19) was purchased from Cell Signaling Technology (Beverly, MA, USA). Rhodamine-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR, USA). Collagen type IA was purchased from Nitta Gelatin (Osaka, Japan). DAF-2/DA was purchased from Daiichi Pure Chemicals (Tokyo, Japan). All other reagents were purchased from Sigma.

Results

TGFβ_1-induced inhibition of cell proliferation in BAECs

TGFβ_1 induced concentration-dependent inhibition of endothelial proliferation, with a concentration of 3 ng ml^{-1} showing a maximal effect (Figure 1a–i). TGFβ_1-induced inhibition of endothelial proliferation was completely abolished by anti-TGFβ_1 antibody (Figure 1a–ii). We therefore used the concentration of 3 ng ml^{-1} in this study and assumed that anti-TGFβ_1 antibody totally neutralized the effects of TGFβ_1.

Expression of smooth muscle markers in TGFβ_1-treated BAECs

Previous reports indicated that TGFβ_1 induced an expression of smooth muscle markers in endothelial cells (Arciniegas *et al*., 1992; Frid *et al*., 2002). Next we examined the expression of smooth muscle markers in control and TGFβ_1-treated BAECs (Figure 1b). Control BAECs showed trace levels of expression of the smooth muscle markers, α-SM actin, calponin and SM myosin. The cells treated with TGFβ_1 for 7 days showed a significant increase in the expression of these marker proteins and these changes were abolished by anti-TGFβ_1 antibody.

TGFβ_1-induced morphological changes in BAECs

Control culture medium did not elicit apparent changes in actin cytoskeleton of BAECs for at least for 7 days (Figure 2a). In contrast, incubation with TGFβ_1 (3 ng ml^{-1}) induced

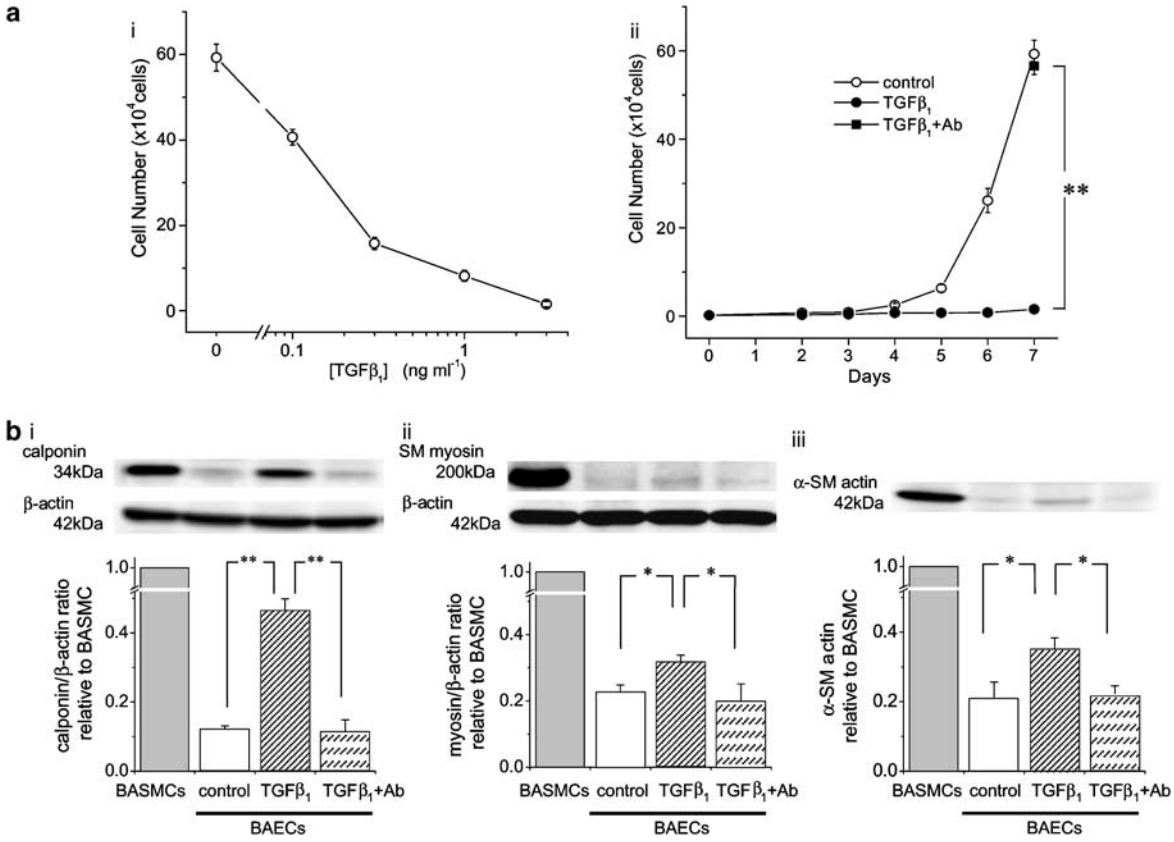

Figure 1 TGFβ_1-induced growth inhibition and expression of smooth muscle markers in BAECs. (**a**) A fixed number of cells (2000 cells) were seeded at day 0, and the cell numbers were manually counted after culturing for 7 days in the absence or presence of various concentrations of TGFβ_1 (i), or for 2–7 days in the absence or presence of 3 ng ml^{-1} TGFβ_1 (ii). In some experiments, anti-TGFβ_1 antibody (300 ng ml^{-1}) was pre-incubated with 3 ng ml^{-1} TGFβ_1 for 1 h at room temperature and applied to the cells (ii, closed square). $^{**}P<0.01$. $n=5$–7. (**b**) Western blotting of the expressions of calponin (i), SM myosin (ii) and α-SM actin (iii) in BASMCs and control, TGFβ_1 (3 ng ml^{-1})-treated and TGFβ_1/antibody-treated BAECs. Expression of β-actin was also measured as an internal control. The cells were cultured in each condition for 7 days. Representative band images are shown in the upper panels. Densitometric analysis of smooth muscle markers/β-actin ratios relative to BASMCs are shown in the lower panels ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$.

actin fibre formation in 1 h in BAECs and these fibres formed by TGFβ_1 became denser in a time-dependent manner, so that peripheral thick actin fibres were observed after 7 days (Figure 2b). Anti-TGFβ_1 antibody completely inhibited the effects of TGFβ_1 on actin fibres (Figure 2c). Dense actin fibres were also observed in BASMCs, but their distribution was not restricted to the peripheral area (Figure 2d).

Contractile properties of TGFβ_1*-treated BAECs*

The results above indicate that TGFβ_1 induces endothelial–mesenchymal transformation in BAECs as previously reported (Arciniegas *et al*., 1992; Frid *et al*., 2002). We then examined the functional alterations of the TGFβ_1-treated BAECs. Previous reports demonstrated that cultured smooth muscle cells exhibit contractility on being embedded in a collagen gel lattice (Kimura *et al*., 2002, 2004). So we used this technique to examine whether TGFβ_1-treated BAECs exhibited similar contractility.

When BASMCs were embedded in a three-dimensional collagen gel lattice, cells showed a spindle shape (Figure 3a–i). In contrast, control BAECs embedded in a similar collagen gel lattice for 3 days showed a capillary-like appearance (Figure 3a–ii). However, when TGFβ_1-treated BAECs were embedded in collagen gel and cultured for 3 days in the presence of TGFβ_1, the cells displayed a smooth muscle-like, spindle shape as shown in Figure 3a–iii. Cells treated with TGFβ_1 and anti-TGFβ_1 antibody showed the capillary-like, and not the spindle shaped, morphology in collagen gels (not shown).

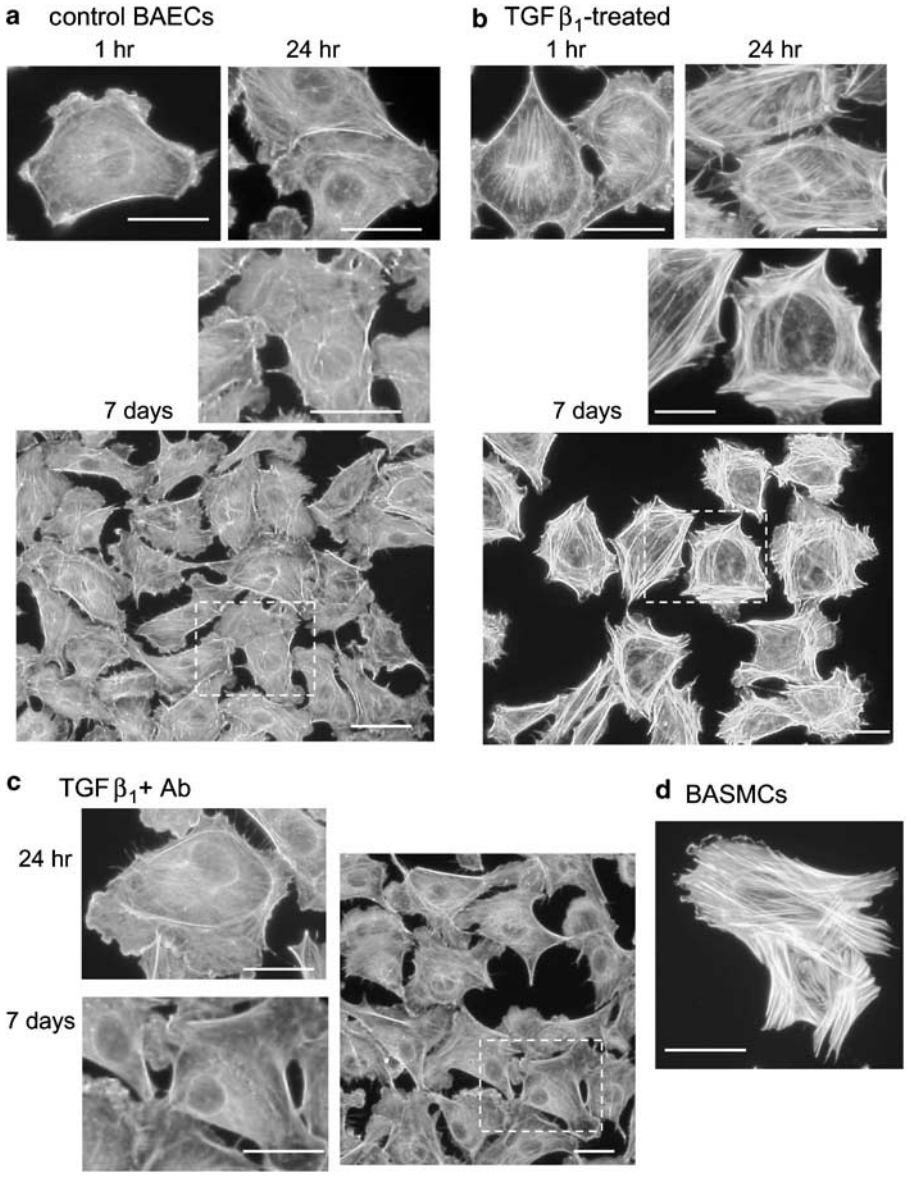

Figure 2 Time-dependent change in actin fibres in BAECs. Control culture medium (**a**) or culture medium containing TGFβ_1 (3 ng ml^{-1}, **b**) or TGFβ_1/antibody (**c**) was applied to subconfluent BAECs. Cytosolic actin cytoskeleton was then stained with rhodamine-phalloidin after 1 h, 24 h or 7 days. Higher magnification images of '7 day' cells are taken from the dotted areas of the corresponding lower magnification images. Actin fibres in BASMCs are also shown (**d**). Scale bar in each panel indicates 50 μm.

ATP (10 μM) induced rapidly developing contraction of gels containing BASMCs. The contraction was maintained during ATP application and reversed to the basal level after the removal of ATP (Figure 3b, open triangles). ATP (10 μM) did not induce any contraction in gels containing control BAECs (Figure 3b, open circles). In contrast, gels with TGFβ_1-treated BAECs showed a slow contraction in response to 10 μM ATP and these gels did not show relaxation for at least 30 min after the removal of ATP (Figure 3b, closed circles). Contraction of TGFβ_1-treated BAECs was not observed without ATP application (not shown). When the embedded BAECs were pretreated with TGFβ_1 in the presence of its neutralizing antibody, gels did not show contraction in response to ATP (Figure 3b, closed squares).

Relaxation responses of TGFβ_1-treated BAECs and BASMCs were further examined with sodium nitroprusside (SNP), an NO donor. In gels containing BASMCs, the sustained contraction induced by ATP was relaxed by SNP in a concentration-dependent manner (Figure 4a, closed triangles). In gels with TGFβ_1-treated BAECs, however, 10 nM SNP did not affect the gradual development of ATP-induced gel contraction, and a partial relaxation was obtained only with a higher concentration of SNP (1 μM; Figure 4a, closed circles).

Endothelin-1 (ET-1, 1 nM) induced a contraction in gels containing BASMCs but not in those with TGFβ_1-treated BAECs (Figure 4b). It is known that both ET_A and ET_B receptors are localized in smooth muscle cells, whereas only ET_B receptor in endothelium, and ET-1 has a higher affinity

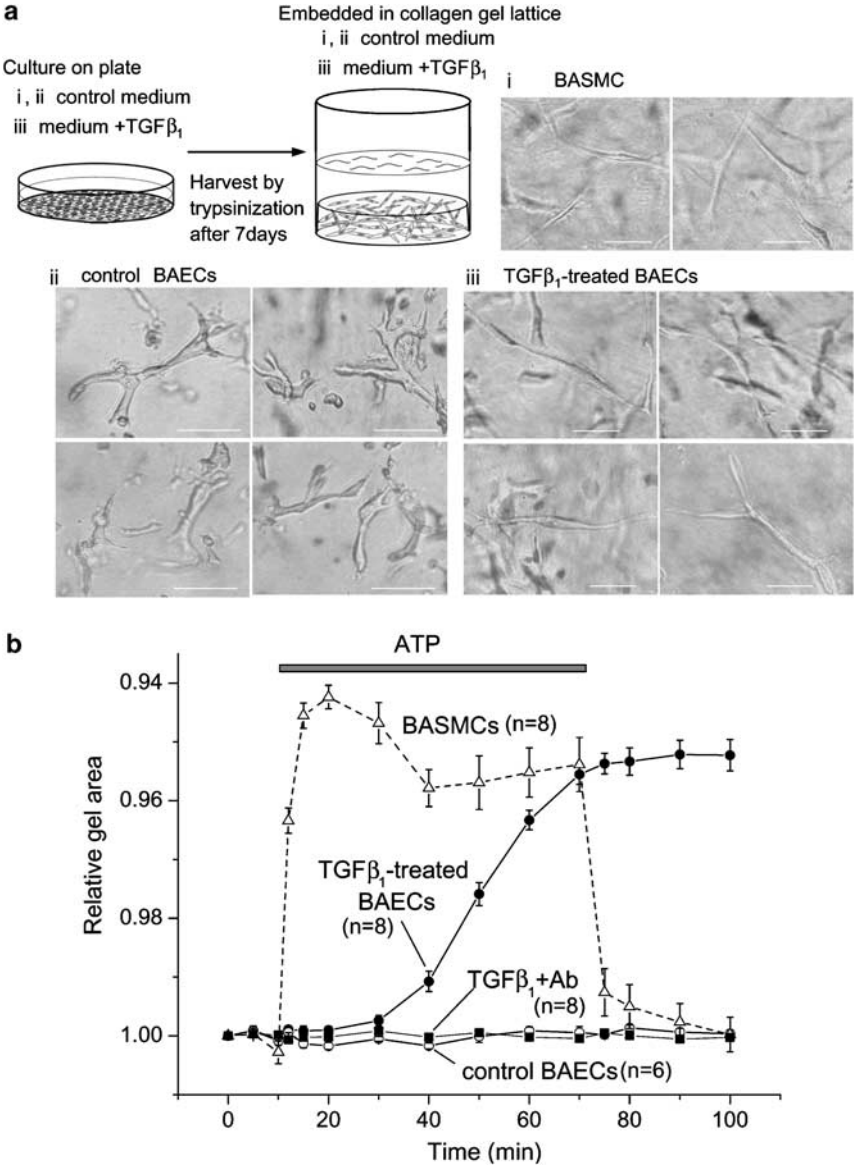

Figure 3 Gel contraction assay. BAECs were cultured on a plate for 7 days in the absence or presence of 3 ng ml^{-1} TGFβ_1, and harvested by trypsinization. Cells were then embedded in collagen gels that were overlaid with the same culture media. BASMCs were also embedded in collagen gels. Cell images in the gels after 3 days are shown in (**a**). Scales, 50 μm. ATP (10 μM)-induced contractions of these gels were assessed by measuring the surface area of the gels (**b**, $n = 6$–8). See Materials and methods for a detailed protocol.

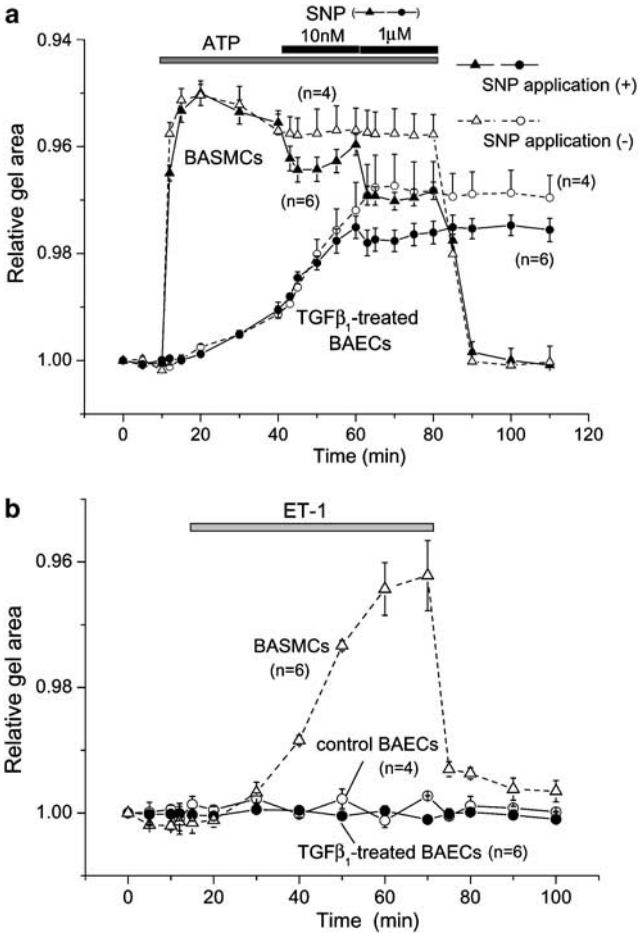

Figure 4 Effects of SNP and ET-1 on gel contraction. (**a**) Gels containing BASMCs or TGFβ_1-treated BAECs were contracted with 10 μM ATP, and the effects of sequential application of 10 nM and 1 μM SNP were examined (closed symbols, $n=4$–6). Control data were obtained without SNP application from the same gel preparation (open symbols, $n=4$–6). Note that 10 nM SNP induced relaxation only in gels containing BASMCs. (**b**) ET-1 (1 nM) induced contraction in gels with BASMCs, but not in gels containing control or TGFβ_1-treated BAECs ($n=4$–6).

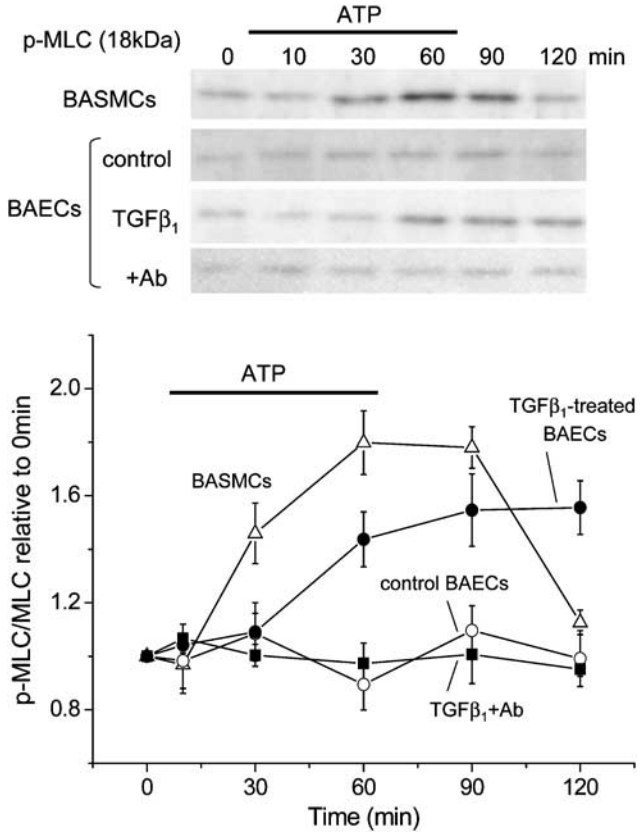

Figure 5 Effects of ATP on p-MLC in BAECs and BASMCs. BAECs were cultured on a plate with control medium or medium containing TGFβ_1 (3 ng ml^{-1}) or TGFβ_1/antibody for 7days. BASMCs were also cultured for 7days. Total cellular proteins were collected before (0 min) or during 10 μM ATP application and after removing ATP at the times indicated. Expression levels of p-MLC and total MLC were then assessed with Western blotting. The upper panels show the representative band images of p-MLC, and the lower panel shows the densitometric analysis of p-MLC/total MLC values relative to the 0 min value ($n=5$).

to ET_A receptor (Schiffrin, 2005). Therefore, we suppose that the absence of ET-1-induced contraction in TGFβ_1-treated BAECs was due to the absence of ET_A receptor expression in BAECs.

Sustained phosphorylation of MLC and reduced expression of MYPT1 in TGFβ_1-treated BAECs

The results above indicate that BAECs acquire contractility by being treated with TGFβ_1. To examine whether the contraction of TGFβ_1-treated BAECs is due to the phosphorylation of contractile proteins or not, we then evaluated the phosphorylation of MLC in the cells that were grown on culture plates and stimulated with 10 μM ATP. BASMCs showed an increase in phosphorylated MLC (p-MLC) in response to 10 μM ATP, which returned to the basal level after the removal of ATP (Figure 5, open triangles). ATP did not induce MLC phosphorylation in control BAECs (Figure 5, open circles). In contrast, BAECs treated with TGFβ_1 for 7 days showed a gradual increase in p-MLC in response to ATP. However, the phosphorylation of MLC was sustained after the removal of ATP in TGFβ_1-treated BAECs (Figure 5, closed circles). ATP did not increase p-MLC in BAECs that were pretreated with TGFβ_1 and its antibody for 7days (Figure 5, closed squares). These results are compatible with the gel contraction assay, and indicate that the contraction of TGFβ_1-treated BAECs was due to the phosphorylation of MLC.

To elucidate the mechanisms of the sustained phosphorylation of MLC, next we evaluated the expression level of MYPT1, the regulatory subunit of myosin light chain phosphatase (MLCP). As shown in Figure 6a, expression of MYPT1 was more abundant in control BAECs than in BASMCs. TGFβ_1 significantly reduced the expression of MYPT1 in BAECs, and anti-TGFβ_1 antibody reversed this effect (Figure 6a). However, ATP did not induce any change in the amount of p-MYPT1 both in control and TGFβ_1-treated BAECs (Figure 6b). Therefore, although inhibitory phosphorylation of MYPT1 augments the contractility in smooth muscle (Feng *et al.*, 1999), the sustained contraction of TGFβ_1-treated BAECs may be mainly due to the reduction of MYPT1 expression but not to its phosphorylation.

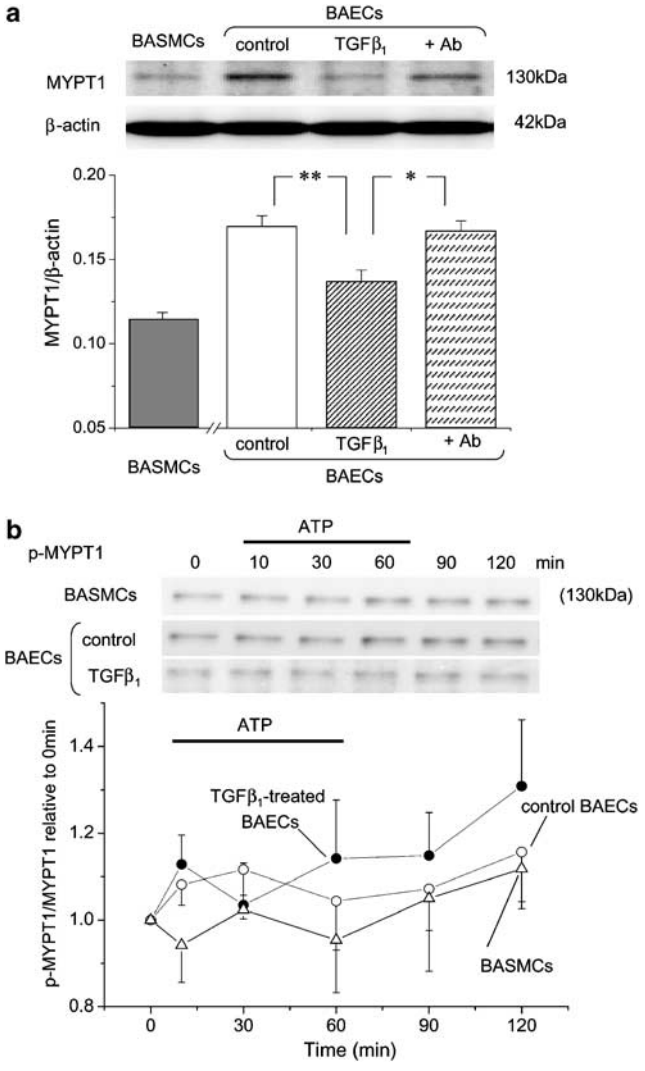

Figure 6 Western blot analysis of the expressions of MYPT1 and p-MYPT1 in BAECs and BASMCs. Total cellular proteins were prepared as described in the legend to Figure 5. Basal expression levels of MYPT1 and β-actin before ATP application are shown in (**a**) band images show data from a representative experiment and the bars show the densitometric analysis of MYPT1 expression relative to β-actin from six experiments (*$P<0.05$, **$P<0.01$). Expression of p-MYPT1 before, during and after ATP (10 μM) application are shown in (**b**). Band images show a representative result and the graph shows the time-dependent change in p-MYPT1/total MYPT values, relative to the initial (time = 0) value ($n=5$).

Calcium mobilizing properties in TGFβ_1-treated BAECs

To further examine the functional alteration of TGFβ_1-treated BAECs, we compared the ATP-induced Ca^{2+} transients in control and TGFβ_1-treated BAECs. Basal $[Ca^{2+}]_i$ was not different between control and TGFβ_1-treated BAECs (control, 106.7 ± 16.9 nM, $n=7$; TGFβ_1-treated, 94.7 ± 16.0 nM, $n=6$). ATP (10 μM) elicited Ca^{2+} transients both in control and TGFβ_1-treated BAECs (Figure 7a). The degree of Ca^{2+} elevation in control BAECs was comparable to that observed in BASMCs in our previous report (Kimura *et al*., 2002). Peak amplitude of Ca^{2+} transients and the time integral of net $[Ca^{2+}]_i$ increment were not different between control and TGFβ_1-treated BAECs (Figure 7b).

ATP-induced NO production in TGFβ_1-treated BAECs

Next, we examined production of NO in TGFβ_1-treated BAECs to assess their endothelial nature. Expression of eNOS protein was not different between control BAECs and the cells treated with TGFβ_1 for 7 days (Figure 8a). Therefore, one can expect that Ca^{2+}-mobilizing stimuli would induce NO production in TGFβ_1-treated BAECs. However, a gradual increase in DAF-2 fluorescence, used as an indicator of NO production (Kojima *et al*., 1998), was induced by 10 μM ATP in control, but not in TGFβ_1-treated BAECs (Figure 8b). ATP-induced increase in DAF-2 fluorescence was restored in the cells treated with TGFβ_1 in the presence of its neutralizing antibody (Figure 8b).

Discussion

We have observed in this study that TGFβ_1 induces the formation of dense actin fibres in BAECs that was time dependent and sensitive to anti-TGFβ_1 antibody (Figure 2). The time course of TGFβ_1-induced actin formation was quite different from that induced by mechanical stress, which is maximally observed in 5 min and converged in 15 min (Koyama *et al*., 2001). Furthermore, mechanical stress-induced actin reorganization is mediated by the RhoA/Rho-kinase pathway (Koyama *et al*., 2001), but we failed to observe the TGFβ_1-induced membrane translocation of RhoA (Watanebe and Oike; unpublished observation). Therefore, TGFβ_1-induced actin fibre formation in BAECs was not due to mechanical stress but to transdifferentiation into smooth muscle-like cells (Arciniegas *et al*., 1992; Frid *et al*., 2002). Also TGFβ_1 increased the expression of the smooth muscle marker proteins, α-SM actin, calponin and SM myosin, in 7 days in BAECs (Figure 1b). Previous reports had shown that TGFβ_1 could induce expression of these smooth muscle markers and had suggested the endothelial–mesenchymal transdifferentiation (Arciniegas *et al*., 1992; Frid *et al*., 2002). Thus, it appears that the experimental conditions that we used in the present study were sufficient to differentiate BAECs into the 'smooth muscle-like cells' previously reported (Arciniegas *et al*., 1992; Frid *et al*., 2002).

This is the first report examining the contractility of TGFβ_1-treated endothelial cells. When TGFβ_1-treated BAECs were embedded in collagen gels, the cells displayed a smooth muscle-like spindle shape (Figure 3a–iii). Also, the gels showed contraction in response to ATP, but the contraction was, in contrast to that observed in gels in which BASMCs were embedded, slow to develop and not reversed by the removal of ATP (Figure 3b). Furthermore, a higher concentration of SNP was required to induce a relaxation in TGFβ_1-treated BAECs than in BASMCs (Figure 4a). These observations indicate that the contractile properties and relaxation responses of TGFβ_1-treated BAECs are different from those of BASMCs. Furthermore, since ET-1 did not induce the contraction of TGFβ_1-treated BAECs (Figure 4b), the ATP-induced contraction of the gels containing TGFβ_1-treated BAECs was not due to contamination with BASMCs.

ATP-induced phosphorylation of MLC in BASMCs and TGFβ_1-treated BAECs, but not in control or TGFβ_1/antibody-treated

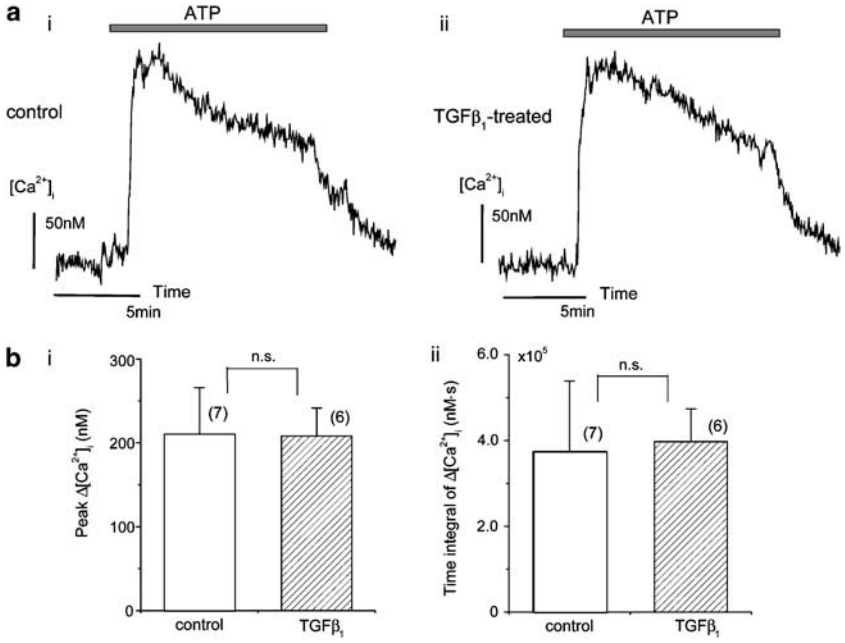

Figure 7 ATP (10 μM)-induced Ca^{2+} transients in BAECs. $[Ca^{2+}]_i$ was measured in control and TGFβ_1-treated BAECs. Ca^{2+} traces from representative cells are shown in (**a**). The bar graphs in (**b**) show the statistical analysis of the peak value (i) and time-integral (ii) of the ATP-induced net $[Ca^{2+}]_i$ increment. Numbers in parenthesis indicate the number of measurements. ns, $P>0.05$.

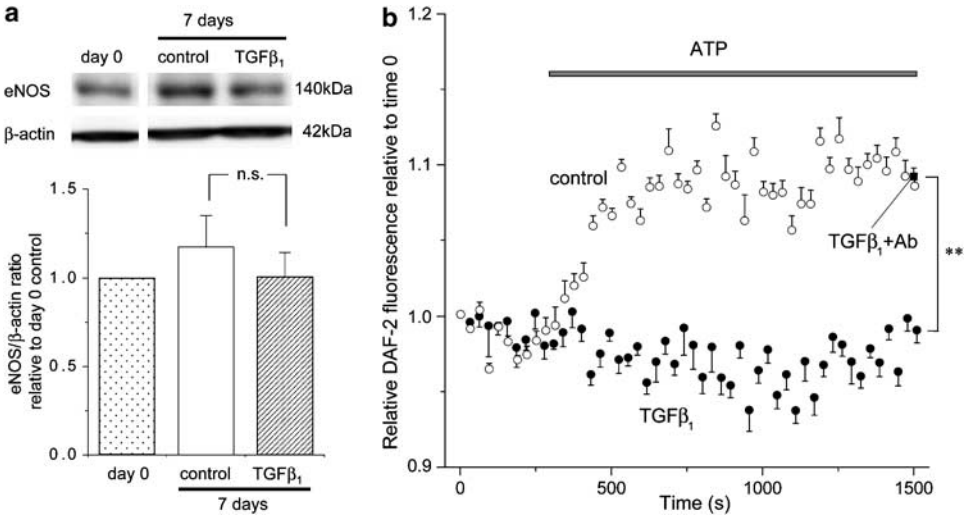

Figure 8 NO production in control and TGFβ_1-treated BAECs. (**a**) Expression of eNOS and β-actin proteins were analysed with Western blotting in control and TGFβ_1-treated BAECs. Band images show representative data and the bar graph shows the mean eNOS/β-actin values, relative to day 0 control ($n=5$). ns, $P>0.05$. (**b**) ATP (10 μM)-induced NO production was assessed with DAF-2 fluorescence. Cells were excited at 490 nm wavelength every 30 s, and fluorescence intensity at 515 nm was measured. DAF-2 fluorescence relative to its initial value (time 0) was then averaged. Open and closed circles show the data from control ($n=22$) and TGFβ_1-treated ($n=20$) BAECs, respectively. Anti-TGFβ_1 antibody restored NO production (closed square, $n=18$). $^{**}P<0.01$, TGFβ_1 vs TGFβ_1/antibody.

BAECs (Figure 5). Although the time course of MLC phosphorylation was slower than that of contraction, we consider this discrepancy was to reflect the fact that the phosphorylation study was performed with the cells seeded on culture plates while the contraction assay used those embedded in collagen gels. MLC phosphorylation did not return to control levels after removal of ATP in TGFβ_1-treated BAECs, and this finding could explain the sustained contraction of the TGFβ_1-treated BAECs in gels. Phosphorylation of MLC is an essential phenomenon for the contraction of smooth muscle cells (Somlyo and Somlyo, 1994) and of non-muscle cells such as fibroblasts (Ehrlich *et al*., 1991; Kolodney *et al*., 1999). In previous reports, contraction of fibroblasts embedded in collagen gels was also shown in response to bovine serum (Kolodney *et al*., 1999), thrombin, ionomycin and lysophosphatidic acid (Emmert *et al*., 2004), but both contraction and MLC phosphorylation were rapid and reversible (Kolodney *et al*., 1999; Emmert

et al., 2004). Therefore, the present results indicate that the contractile properties of TGFβ_1-treated BAECs are not identical to those of the smooth muscle cells or fibroblasts.

Previous reports suggested that endothelial–mesenchymal transdifferentiation may be involved in the repair process of injured mature vessels as the source of luminal cells (Arciniegas *et al*., 1992; Frid *et al*., 2002). However, because TGFβ_1-treated BAECs showed impairment of relaxation responses, such transdifferentiated cells could not restore all the functions of damaged vessels, in which relaxation is as important as contraction. We have shown that TGFβ_1 reduced the expression of MYPT1, a catalytic subunit of MLCP (Pfitzer, 2001), in BAECs (Figure 6a). As ATP did not increase p-MYPT1 in TGFβ_1-treated BAECs (Figure 6b), we would postulate that the reduced expression of MLCP but not its inhibitory phosphorylation might be, at least partially, responsible for the sustained contraction of TGFβ_1-treated BAECs. We did not expect that the expression level of MYPT1 would be higher in control BAECs than in BASMCs (Figure 6a), but this may be because MLCP plays an important physiological role in controlling barrier function in intact endothelial cells (Verin *et al*., 2000). Reduction of MYPT1 levels by TGFβ_1 could thus lead to the impairment of barrier function, one of the essential functions of endothelium, in injured or atherosclerotic vessels. The expression levels of other MLCP subunits such as PP1c (Ito *et al*., 2004) have not been examined in this study, so it should be noted that the quantitative and/or functional alteration of any of these subunits may also be involved in the pathogenesis of the impaired relaxation in TGFβ_1-treated BAECs.

Previous reports indicated that TGFβ_1 acutely increased the expression of eNOS protein and NO production in endothelium (Inoue *et al*., 1995; Saura *et al*., 2002; Tai *et al*., 2004). These authors speculated that TGFβ_1 generated in injured vessels or atherosclerotic lesions would increase NO production, thereby relaxing vessels to protect them from increased flow. However, in the present study, TGFβ_1-treated BAECs lost their ability to produce NO in 7 days even though expression of eNOS protein and Ca^{2+} mobilization were intact (Figures 7 and 8). Although TGFβ_1 inhibits cell proliferation, this does not explain the reduced NO production in TGFβ_1-treated BAECs, because we measured fura-2 and DAF-2 fluorescence from isolated non-confluent cells with similar cell densities both in control and TGFβ_1-treated BAECs. Saura *et al*. (2002) reported that TGFβ_1 activated eNOS promoters via Smad2 translocation, but this was observed only during 2–6 h after TGFβ_1 treatment. So we suppose that the TGFβ_1-induced upregulation of eNOS, if present, is transient, and the reduction of NO production observed in the present study was not due to the alteration of eNOS expression. In human endothelium, TGFβ receptors localize in caveolae, where eNOS protein is also located and generates NO (Minshall *et al*., 2003), and TGFβ_1 rapidly expels eNOS from caveolae thereby inactivating eNOS (Schwartz *et al*., 2005). This mechanism may explain the present results and we conclude that mesenchymally transformed BAECs would lose another central endothelial function, the generation of NO.

The increased secretion of TGFβ_1 in injured vessels was reported to continue for up to 2 weeks (Kanzaki *et al*., 1995). Therefore, we believe that chronic effects of TGFβ_1, as shown in the present study, would have more pathophysiological significance than its acute actions within a few hours. It was reported that anti-TGFβ antibody accelerated atherosclerosis in apoE knockout mice (Mallat *et al*., 2001), and an atheroprotective role of TGFβ_1 has been suggested (Grainger, 2004). However, another group observed that TGFβ_1 signalling in T-cells but not in the vascular wall played a role in protecting vessels from arteriosclerosis in apoE knockout mice (Robertson *et al*., 2003).

The present results firstly suggest that TGFβ_1-treated, mesenchymally transdifferentiated endothelial cells would not be capable of substituting fully for injured vascular smooth muscle cells, because the cells would not possess sufficient relaxing ability. Furthermore, the absence of NO production in TGFβ_1-treated endothelium would exclude a simple protective role for TGFβ_1 through increased NO production in injured and/or atherosclerotic vessels. Previous reports have suggested another possibility for the roles of endothelial–mesenchymal transdifferentiation; that is, non-muscle epithelioid cells are dominant in neointima after injury (Myit *et al*., 2003), and involved in intimal thickening and pulmonary vascular remodeling in the embryonic arteries (Arciniegas *et al*., 2005). In conclusion, TGFβ_1-induced endothelial–mesenchymal transdifferentiation is not, of itself, beneficial for the maintenance of vascular integrity, and therefore may have other consequences for vascular remodelling that have not been clarified in the present study.

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Conflict of interest

The authors state no conflict of interest.

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