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Ambient pressure stimulates immortalized human aortic endothelial cells to increase DNA synthesis and matrix metalloproteinase 1 (tissue collagenase) production

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Abstract In the present study, we investigated the effect of ambient pressure on [^{3}H]-thymidine incorporation and on the production of matrix metalloproteinase 1 (tissue collagenase/proMMP-1) using human aortic endothelial cells immortalized with simian virus 40 (SE-1). Incubation of cells at ambient pressures of 50 and 100 mmHg for 24 h slightly increased [^{3}H]-thymidine incorporation when directly compared with normal culture conditions. The amount of [^{3}H]-thymidine incorporated in SE-1 reached a maximum at 150 mmHg, while a further increase in pressure to 200 mmHg decreased incorporation. The same ambient pressure slightly stimulated human aortic intimal smooth muscle cells (SMC) to increase [^{3}H]-thymidine incorporation but not medial SMC. Immunoblot analysis also showed that ambient pressure, ranging from 50 to 200 mmHg, like 12-O-tetradecanoyl-phorbol-13-acetate stimulated SE-1 to produce proMMP-1, an effect not seen with either intimal or medial SMC. The amount of proMMP-1 produced also reached a maximum level at 150 mmHg. We postulate that human endothelial cells are ambient pressure sensitive and that relatively lower ambient pressures play an important role in the growth of endothelial cells, while higher pressures injure endothelial cells, resulting in the initiation of atherosclerosis. This cell line may prove useful in the investigation of both the physiological and pathological roles of blood pressure on endothelial cell function.

Key words Pressure · Human aortic endothelial cells
Matrix metalloproteinase 1 · Angiogenesis
Atherosclerosis

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Introduction

Shear stress, a physiological overload on vascular endothelium, acts mainly in a horizontal direction in blood vessels. It induces not only morphological changes [42], but also causes functional modifications in endothelial cells [6, 8, 11, 41]. It has been observed that early atherosclerotic lesions arise in areas exposed to low shear stress [3]. Interestingly, it has been reported that shear stress also increases the metabolism of inositol phosphate (PI turnover) and intracellular $[Ca^{2+}]_i$ level in endothelial cells [4]. In terms of blood pressure, in which loads act vertically to the vascular wall, however, an appropriate in vitro experimental model has not been established, and furthermore, little is known about its effects on the metabolism of endothelial cells.

In our previous study [29] a human aortic endothelial cell line immortalized with simian virus 40 was established. This cell line can produce matrix metalloproteinase 1 (tissue procollagenase/proMMP-1), an enzyme which degrades extracellular matrix macromolecules [36]. Since the synthesis and degradation of extracellular matrix macromolecules is an essential step for reproduction [10, 26], morphogenesis [9, 22], tissue resorption [13–15, 37, 38] and many pathological processes including tissue remodelling [16, 24], atherosclerosis [30], and movement of malignant neoplasms [20, 29, 33] matrix-degrading enzymes may play an important role in all these processes. We have previously reported [30, 39] that human aortic smooth muscle cells (SMC) produce proMMPs in relation to cell proliferation and development of the aorta, suggesting that some matrix metalloproteinases are important factors for cell growth in the aortic tissue. In contrast, dexamethasone inhibits both DNA synthesis and the production of MMPs in SMC treated with platelet derived growth factor (PDGF) [18]. Therefore, it is possible that some MMPs may play an important role in both destruction and repair of endothelial cells at sites injured by a variety of factors, including blood pressure.

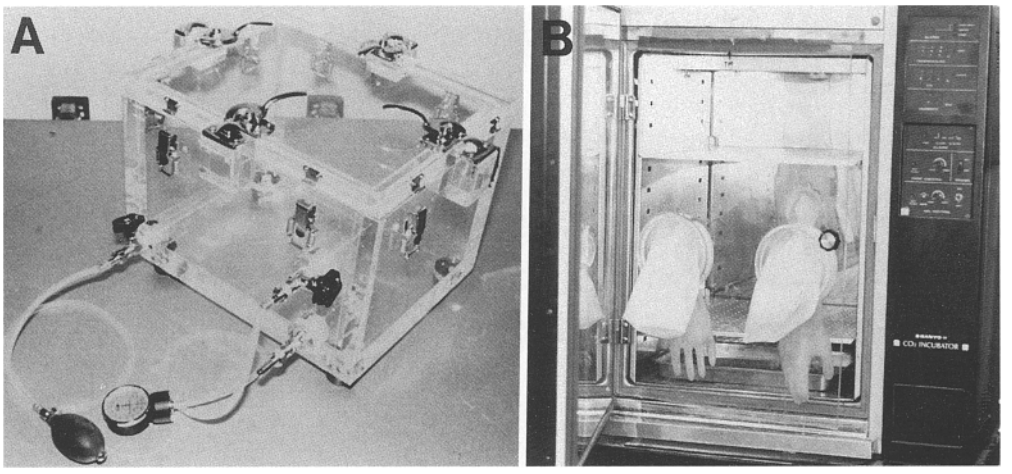

Fig. 1 A pressure chamber with a haemodynamometer (**A**) and a CO_2 incubator with gloves (**B**)

In the present study, in order to investigate the effects of a vertical load of blood pressure on endothelial cells in vitro, we have examined the effects of ambient pressure on DNA synthesis and on proMMP-1 production by the immortalized human aortic endothelial cell line (SE-1) in vitro.

Materials and methods

For cell culture, a pressure chamber made of hard plastic plate with a haemodynamometer (Fig. 1A), measuring 40×28×25 cm, was set in a CO_2 incubator at 37° C in an atmosphere of 5% CO_2–95% air mixture for 10 min. As shown in Fig. 1B, the cell incubator has a plastic door with gloves so that operations inside the cell can be carried out without opening the door. The air in the closed pressure chamber, which is the same as that in the incubator, can be pressurized from 0 to 200 mmHg for several weeks. SE-1 and primary cultures of human aortic intimal SMC were isolated from human aortas as described previously [21].

SE-1 and human aortic SMC were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Co., Tokyo) containing 10% fetal bovine serum (FBS) in 6-well plates. The medium was then changed for serum-free or serum-containing fresh medium and supplemented with 1 μCi/well of [^{3}H]thymidine (Amersham, Buckinghamshire, UK). The cells were subsequently incubated in the chamber (Fig. 1A) at various ambient pressures for 24–48 h to analyse the effects of such pressure on [^{3}H]thymidine incorporation and production of MMPs in the cells. After removal of the medium, the cells were washed with phosphate buffered saline (PBS) and were dissolved in 0.5 M NaOH and neutralized with an equal volume of 0.5 M HCl. Each sample was then absorbed onto a mixed cellulose acetate-cellulose nitrate filter (Millipore, Bedford, Mass.). After washing with 10% trichloroacetic acid, radioactivity on the filter was determined by liquid scintillation spectroscopy (LSC673, Aroka, Japan), as described previously [16].

To check for any changes in the pH of the culture medium, serum-free conditioned medium which had been incubated in the chamber under ambient pressure was immediately harvested into a syringe and its pH measured by Chiba Corning 178 pH/Blood Gas Analyser.

After the cells had reached confluence in 35-mm Petri dishes, 1 ml serum-free medium was added to each dish. The cells were then incubated under normal culture conditions or in the chamber at various ambient pressures for 48 h. The serum-free medium was taken and used for further experiments. For immunoblotting, proteins separated by 10% SDS-PAGE were transferred onto a nitrocellulose filter. Reconstituted non-fat dried milk (20%, W/V) was used as a blocking agent, as described previously [29, 39]. The filters were treated with sheep anti-(human proMMP-1) serum for 1 h. After repeated washing with PBS, the filters were incubated with peroxidase-conjugated rabbit anti-(sheep IgG)IgG for 1 h. Antigens were visualized as described previously [29, 39]. The antibody against human proMMP-1 was donated by Dr. H. Nagase [29, 39]. The effect of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and dexamethasone on the production of proMMP-1 by SE-1 at an ambient pressure was also checked. After reaching semi-confluency in 6-well plates, 1 ml serum-free medium containing 10 ng/ml TPA or 10^{-6} M dexamethasone was added to the cultures which were incubated for a further 2 days. After incubation with these agents, the medium was harvested and stored at −70° C until used in immunoblot analysis. Bands corresponding to proMMP-1 were counted for the density by an image analyser (IBAS; Zeiss, Jena, Germany) and the amount of proMMP-1 was calculated as a percentage of the control amount.

For immunocytochemistry the cells were cultured on cover slips, incubated with 1 μg/ml monensin, which inhibits protein secretion by blocking the Golgi apparatus, prior to fixation with 95% cold acetone for 1 min, and dried at room temperature. The cells were incubated with the first antibodies for 1 h. After washing with PBS, the cells were incubated with fluorescence-conjugated second antibodies against sheep IgG for 1 h. The specimens were observed with an Olympus fluorescence microscope.

Results

Before the medium was used in cell culture, its pH was adjusted to 7.4 with 7.5% sodium bicarbonate. Since any changes in pH would affect cell proliferation, pH levels in the medium were checked immediately after the incubation at ambient pressures. The results are shown in Table 1. Incubation at 0, 150 and 200 mmHg for 24 h and 150 mmHg for 48 h did not change the pH of the medium, indicating that the pH of the medium was constant at ambient pressure and therefore suitable for cell growth.

Incubation of SE-1 in serum-containing DMEM at ambient pressures of 50 or 100 mmHg for 24 h resulted in a slight increase in the amount of [^{3}H]-thymidine incorporated when compared with the control incubations (0 mmHg). Increase in the pressure to 150 mmHg significantly increased [^{3}H]-thymidine incorporation in SE-1 incubated in medium both with and without serum. However, when the pressure was increased to 200 mmHg, in-

Table 1 Effect of ambient pressures on pH

Ambient pressure	pH
0 mmHg (24 h)	6.789±0.037
150 mmHg (24 h)	6.722±0.010
150 mmHg (48 h)	6.769±0.082
200 mmHg (24 h)	6.772±0.014
	mean±SD

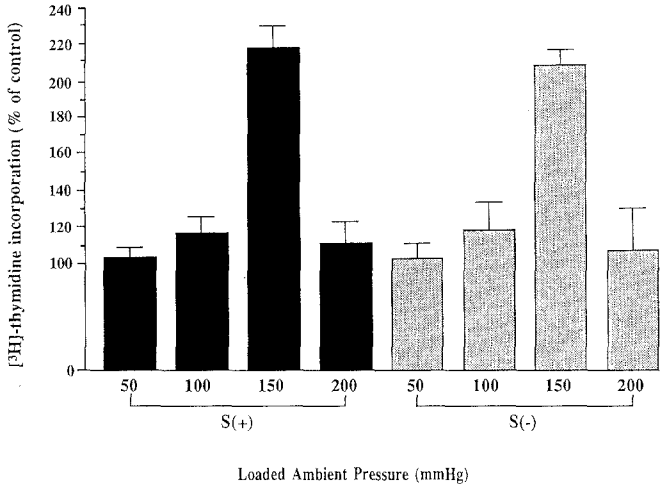

Fig. 2 Effect of ambient pressures on [^{3}H]-thymidine incorporation. After incubation at ambient pressures ranging from 0 to 200 mmHg for 24 h, the amount of [^{3}H]-thymidine incorporated by SE-1 was measured. The values (mean±SE, n=3) are expressed as a percentage of the control (0 mmHg; 100%) at each point from experiments done in triplicate. *Black bar* Medium containing 10% serum, *grey bar* serum-free medium, *S*(+) serum-containing medium, *S*(–) serum-free medium

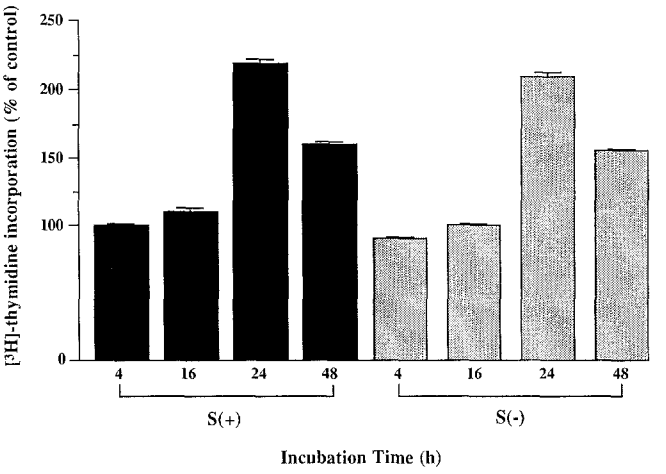

Fig. 3 Effect of incubation time at ambient pressures on [^{3}H]-thymidine incorporation. Incubation at a pressure of 150 mmHg for 4 or 16 h had no effect on [^{3}H]-thymidine incorporation in endothelial cells. After incubation at an ambient pressure for 24 h, SE-1 showed an increase in cell proliferation. A further increase in incubation time to 48 h resulted in a decrease of the amount of [^{3}H]-thymidine incorporated. The values (mean±SE, n=3) are expressed as a percentage of the control (0 mmHg: 100%) at each point from experiments done in triplicate. *Black bar* Medium containing 10% FBS, *grey bar* serum-free medium, *S*(+) serum-containing medium, *S*(–) serum-free medium

corporation decreased to the level observed at 50 or 100 mmHg. In Fig. 2, the results are expressed as a percentage of the control at each pressure level. Similar results were observed in experiments conducted in serum-free medium.

Experiments performed to observe the relationship between incubation time (4–48 h) at a pressure of 150 mmHg and [^{3}H]-thymidine incorporation in SE-1 are shown in Fig. 3. Maximum up-regulation of [^{3}H]-thymidine incorporation in serum-containing DMEM was found after incubation at an ambient pressure of 150 mmHg for 24 h. The level of cell proliferation decreased after incubation for 48 h under the same pressure load. Similar results were observed in experiments conducted in serum-free medium.

The effect of ambient pressures on [^{3}H]-thymidine incorporation in human aortic intimal and medial SMC in serum-free DMEM was compared with that of SE-1 (Table 2). Human intimal SMC also showed a slight but significant response to a pressure of 150 mmHg for 24 h, even though the amount of [^{3}H]-thymidine incorporation was markedly lower than that seen in SE-1. In contrast, in human medial SMC it was not stimulated by ambient pressure. Again, the presence or absence of serum in the culture medium made no difference to the degree of [^{3}H]-thymidine incorporation observed.

As shown in Fig. 4, ambient pressure stimulated SE-1 to produce proMMP-1. Maximum up-regulation of the production of proMMP-1 by SE-1 in serum-free DMEM was found after incubation at an ambient pressure of 150 mmHg for 24 h. The production of proMMP-1 by intimal or medial SMC was not stimulated by ambient pressure. After treatment with 1 μg/ml monensin, immunofluorescence staining showed an ambient pressure of 150 mmHg to stimulate SE-1 to synthesize proMMP-1 (Fig. 5).

Dexamethasone (10^{-6} M) inhibited the production of proMMP-1 by SE-1 at ambient pressure (Fig. 6B). In contrast, 10 ng/ml TPA stimulated the production of proMMP-1 by SE-1 (Fig. 6C). However, ambient pressure and TPA did not stimulate the production of proMMP-1 by SE-1 in the presence of 10^{-6} M dexamethazone (Fig. 6D).

Discussion

Tokunaga et al. [35] reported that some levels of ambient pressure promoted growth of human umbilical cord vein endothelial cells, whereas high levels of pressure injured the cells. The results of our study also suggest that ambient pressures up to 150 mmHg stimulate [^{3}H]thymidine incorporation of SE-1, and that an increase in the ambient pressure to 200 mmHg inhibits it, to the level seen in control incubations. Up-regulation of [^{3}H]-thymidine incorporation in the cells was seen after incubation for 24 h at 150 mmHg. An increase in the incubation time to 48 h

Table 2 [^{3}H]-thymidine incorporation (DPM×10^{-3}/dish) *SE1* immortalized human aortic endothelial cells, *ISMC* Human aortic intimal smooth muscle cells, *MSMC* human aortic medial smooth muscle cells

	SE1		ISMC		MSMC	
Pressure (mmHg)	0	150	0	150	0	150
	41.543	82.490	19.217	24.473	7.609	7.602
	35.671	86.426	18.501	23.193	7.573	7.241
	34.087	79.278	17.983	20.091	7.036	7.168
Mean±SD	37.100±3.21	82.731±2.92	18.567±0.506	22.585±1.83	7.406±0.26	7.337±0.19

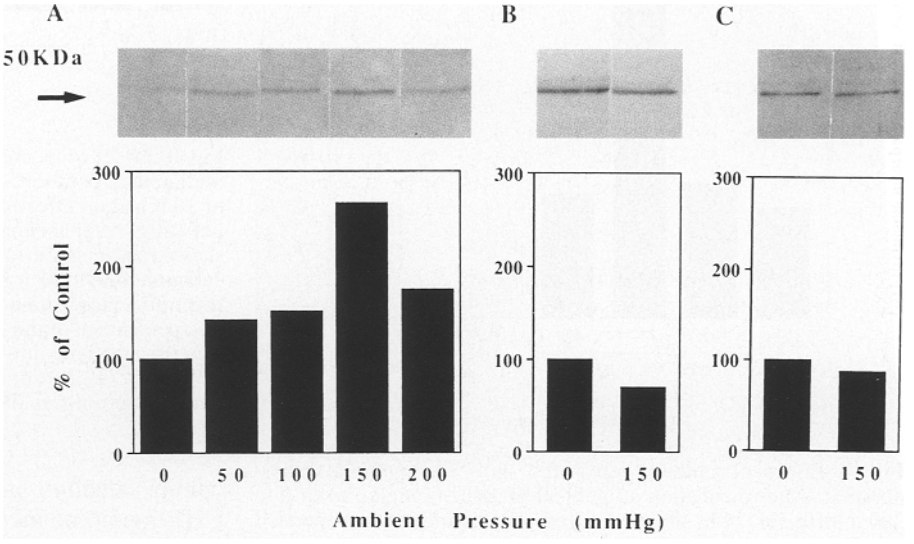

Fig. 4A–C Immunoblot analysis of the effect of ambient pressure on proMMP-1 production. Various degrees of ambient pressure were used for 24 h to stimulate SE-1 to produce proMMP-1 of M_r=53000 up to a maximum level of 150 mmHg (**A**). However, production of proMMP-1 by human aortic intimal smooth muscle cells in (SMC) (**B**) or medial SMC (**C**) was not increased clearly at an ambient pressure of 150 mmHg. *Bars* represent the values measured by an image analyser and calculated as a percentage of the control amount (0 mmHg: 100%)

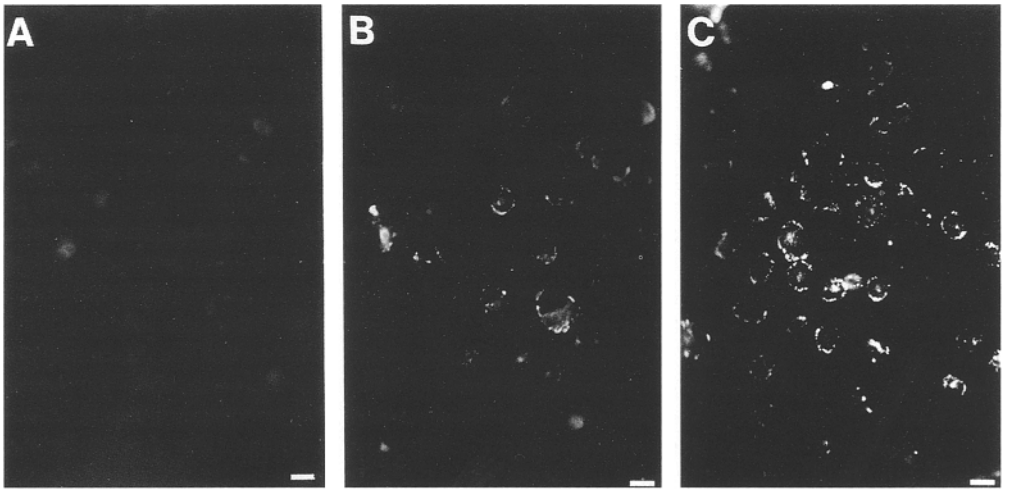

Fig. 5A–C Immunocytochemical staining. After treatment with monensin, immunofluorescence micrographs dramatically demonstrate that production of proMMP-1 by SE-1 was stimulated at an ambient pressure of 150 mmHg (**C**). **A** Control, untreated cells; **B** incubated cells at an ambient pressure of 150 mmHg without treatment with 1 μg/ml monensin; **C** incubated cells at an ambient pressure of 150 mmHg and treated with 1 μg/ml monensin

decreased the amount of [^{3}H]-thymidine incorporated. Since physiological pressure is pulsatile, our pressure chamber system is not itself physiological; constant pressure is used. However, ambient pressure is a factor involved in blood pressure and these results nevertheless suggest that relatively low ambient pressures stimulate endothelial cell growth, whereas higher pressures can injure cells.

It is well known that injury to endothelial cells is one of the essential steps in the initiation and progression of atherosclerosis [27] and it has been suggested that the ambient pressure may play an important role in physiological and pathological degeneration and regeneration of endothelial cells. The inhibition of DNA synthesis seen after incubation of SE-1 in vitro for 24 at 200 mmHg or for 48 h at 150 mmHg may reflect a reduction in their viability, and thus might be interpreted to suggest that high levels of ambient pressure initiate atherosclerosis. However, ambient pressure levels of approximately 150 mmHg may play an important role in the repair of injured endothelium. In contrast, ambient pressure did not stimulate SMC to proliferate, even though DNA synthesis in

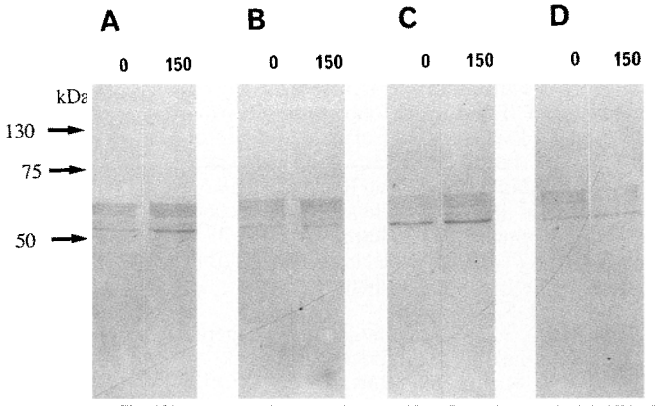

Fig. 6A–D Effects of dexamethasone and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on proMMP-1 production by SE-1 at ambient pressure. **A** Ambient pressure of 150 mmHg, **B** ambient pressure $+10^{-6}$ M dexamethasone, **C** ambient pressure +10 ng/ml TPA, **D** ambient pressure +10 ng/ml TPA+10^{-6} M dexamethasone

the intimal SMC was slightly increased at 150 mmHg for 24 h. The ambient pressure does not apparently play a role in the proliferation of SMC in atherosclerosis.

The turnover of extracellular matrix macromolecules plays an important role in tissue remodelling and atherosclerosis [25, 27]. In a previous study [31, 39], we reported that PDGF-, epidermal growth factor (EGF)-, and TPA-stimulated SMC produce proMMP-1. This enzyme, which can degrade collagen types I, II, and III [23, 36], was produced in the thickened aortic intima [30]. Since PDGF also regulates the proliferation of SMC and the production of extracellular matrix macromolecules in atherosclerotic foci [28], the production of proMMP-1 is likely to be closely related to SMC migration and proliferation. However, we have also reported that SE-1 can also produce proMMP-1 [29], and that this is stimulated by TPA. Therefore, in the present study, we examined the effect of ambient pressure on the production of proMMP-1 by endothelial cells. Our results show that proMMP-1 production was stimulated in a dose-dependent manner with the increase in ambient pressure from 50 to 150 mmHg. However, at a pressure of 200 mmHg, proMMP-1 production by SE-1 decreased to a level below that seen at 150 mmHg. These results parallel those seen for DNA synthesis. Therefore, it can be seen that exposure of endothelial cells to varying levels of ambient pressure results in the production of proMMP-1 as well as their replication. The production of proMMP-1 may be responsible for the remodelling of the endothelial bed in areas where there is ongoing repair of the arterial wall. Tokunaga et al. [35] also reported that prostacyclin production by endothelial cells is highest when the cells are cultured in atmospheric pressure, whereas ambient pressure inhibits the production. We also found that prostacyclin inhibits proMMP-1 production by SMC [18]. Therefore, a decrease in production of prostacyclin may be one of factors that stimulate proMMP-1 production by SE-1.

The mechanism by which SE-1 responds to ambient pressure is unknown. In our experiments with human aortic SMC, we found that PDGF, EGF and TPA stimulated the production of proMMP-1 [31, 39]. This effect of PDGF in SMC probably depends on its binding to a specific receptor, thereby activating the phosphatidyl inositol pathway (PI turnover) and causing an increase in cytosolic $[Ca^{2+}]_i$, which ultimately leads to activation of C-kinase [17]. However, unlike the SMC, SE-1 as well as normal endothelial cells do not express PDGF receptors on their cell surfaces (data not shown). Therefore, PI turnover may not play a key role in proMMP-1 production in endothelial cells. Recent studies have suggested that like EGF, PDGF is also an activator of mitogen-activated protein kinase (MAP kinase) [12], suggesting that MAP kinase may also play an important role in the production of proMMP-1 by human aortic SMC. However, TPA also activates diacylglycerol, an intermediate in PI turnover [5, 7, 19]. The gene of proMMP-1 contains a 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE), which also acts as a promoter region for the protein kinase C signal transduction pathway, and binds to the nuclear factor AP-1. The oncoproteins *jun* and *fos* cooperate in binding to the TRE, resulting in up-regulation of many genes, including the gene for proMMP-1 [1, 2]. Steroids and retinoic acid control various metabolic processes in a wide range of cells by binding to specific intracellular receptors, thereby repressing the expression of specific target genes [32, 34]. In addition, it has been suggested that these agents make a complex with the c-*jun* protein, which then loses its ability to bind to promoter sequences such as the AP-1 site, and therefore may result in the inhibition of transcription of the proMMP-1 gene [40]. We have previously described how dexamethasone inhibited proMMP production by SMC [18]. In the present study, dexamethasone gave similar results at different ambient pressures to those in the previous study. Therefore, we cannot discount the possibility that proMMP-1 production by SE-1 in response to ambient pressures may involve diacylglycerol and/or cytosolic $[Ca^{2+}]_i$ as second messengers.

In conclusion, our data suggest that ambient pressure may be responsible for the repair of injured endothelium by stimulating endothelial cell replication. However, either high levels of pressure and/or long-term loading may cause endothelial injury or inhibit the repair of the cells, leading to the development of atherosclerosis. An ambient pressure of 150 mmHg was found to stimulate maximally both endothelial cell proliferation and function, including the production of proMMP-1. SE-1 will probably prove useful for studies investigating the effects of blood pressure on endothelial cell function.

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