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Role of Transforming Growth Factor-β1 on Platelet-Induced Enhancement of Endothelin-1 Production in Cultured Vascular Endothelial Cells[1]

SATOSHI MURATA, YASUO MATSUMURA, KIMI TAKADA, YUKARI ASAI, MASANORI TAKAOKA and SHIRO MORIMOTO

Department of Pharmacology, Osaka University of Pharmaceutical Sciences, 2–10-65 Kawai, Matsubara, Osaka 580, Japan

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ABSTRACT

We and other investigators obtained evidence that platelets stimulate endothelin-1 (ET-1) production at both message and protein levels in vascular endothelial cells (ECs), and that platelet-derived transforming growth factor-β1 (TGF-β1) is responsible for this stimulation. In the present study, we examined the effects of acidification or heat treatment, known to activate latent TGF-β1, on the platelet supernatant-induced ET-1 production in cultured porcine aortic ECs. Supernatant of platelets (6.0×10^8 platelets/ml) aggregated by adenosine diphosphate contained large amounts of TGF-β1, but were almost in a latent form, and the proportion of active TGF-β1 in the supernatant was increased markedly in the case of acidification or heat treatment. These treatments also significantly potentiated the supernatant-induced stimulation of prepro ET-1 mRNA expression and the ET-1 release in ECs. Purified TGF-β1 also enhanced ET-1 release, dose-dependently, but the enhancement declined at the higher concentrations. Thus, powerful stimulation of ET-1 production by platelet supernatant after acidification or heat treatment cannot be explained only by increments in active TGF-β1. The supernatant-induced stimulation of ET-1 synthesis was significantly inhibited by concomitant treatment of TGF-β1 neutralizing antibody, but this inhibition was incomplete even at a concentration that abolished TGF-β1-induced maximal stimulation. These results suggest that platelet-induced stimulation and subsequent acidification and heat treatment-induced potentiation on endothelial ET-1 production depend closely on release and activation of TGF-β1 derived from platelets. However, when TGF-β1 concentration is increased, this peptide may further stimulate ET-1 production, probably through interactions with other platelet-derived substances.

ET-1 was first isolated and identified as a 21-amino acid residue potent vasoconstrictor peptide in the supernatant of cultured porcine aortic ECs (Yanagisawa *et al.*, 1988). This peptide has a wide variety of actions on both vascular and nonvascular tissues (Yanagisawa *et al.*, 1988; Yanagisawa and Masaki, 1989) and might participate in certain pathological states such as pulmonary hypertension (Stewart *et al.*, 1991), disseminated intravascular coagulation (Asakura *et al.*, 1992) and cerebral vasospasm after subarachnoid hemorrhage (Matsumura *et al.*, 1991). It has been reported that agents including thrombin, angiotensin II, arginine-vasopressin and TGF-β1 induce the expression of ET-1 message (Yanagisawa *et al.*, 1988; Hahn *et al.*, 1990; Tomobe *et al.*, 1993) and increase ET-1 release in ECs (Kurihara *et al.*, 1989; Boulanger and Lüscher, 1990; Emori *et al.*, 1991).

Ohlstein *et al.* (1991) found that platelets enhanced the expression of ET-1 mRNA and ET-1 biosynthesis in cultured ECs. Subsequently, we reported that the supernatant of platelets also has similar stimulatory effects on endothelial ET-1 production, and these supernatant-induced actions were suppressed significantly by treatment with TGF-β1 neutralizing antibody (Matsumura *et al.*, 1994; Umekawa *et al.*, 1994). Thus, we conclude that platelets stimulate endothelial ET-1 production mainly through the release of TGF-β1. Because the interaction between platelets and ECs plays a key role in induction of various vascular diseases such as atherosclerosis and thrombosis (Ware and Heistad, 1993), the platelets-induced enhancement of ET-1 production in ECs may contribute to the genesis and/or maintenance of these diseases.

TGF-β1, a 25 kD homodimeric polypeptide, is secreted from ECs, smooth muscle cells and platelets (Assoian and Sporn, 1986; Sato *et al.*, 1993). However, this molecule is secreted predominantly as a biologically inactive latent form that is unable to bind its receptors (Lawrence *et al.*, 1985;

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ABBREVIATIONS: ET, endothelin; ECs, endothelial cells; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; IgG, immunoglobulin G; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDGF, platelet-derived growth factor.

Miyazono *et al.*, 1988). Therefore, the activation step after secretion of this latent form is essential for expression of biological activities. Although the *in vivo* mechanisms for the activation of latent TGF-β1 are unknown, various chemical and enzymatic treatments such as extreme pH values, heating and plasmin activate the latent TGF-β1 to mature TGF-β1 *in vitro* (Lyons *et al.*, 1988; Miyazono *et al.*, 1988).

To better understand the mechanisms of platelet-induced augmentation of endothelial ET-1 production, the present study was designed to examine the role of TGF-β1 on platelet-induced enhancement of ET-1 production in cultured vascular ECs, by using chemical activation of platelet-derived latent TGF-β1.

Methods

Preparation of platelet supernatant. Blood taken from the abdominal aorta of Sprague-Dawley rats (Japan SLC Inc., Shizuoka, Japan) into tubes containing 0.38% trisodium citrate was centrifuged at 200 × *g* for 10 min at room temperature. Platelet-rich plasma was then collected and centrifuged at 750 × *g* for 15 min. The pellet was suspended in 12 mM Tris-HCl buffer containing 139 mM NaCl (pH 7.4), and the suspension was centrifuged at 600 × *g* for 10 min. Finally, the pellet was resuspended in DMEM supplemented with 0.01% bovine serum albumin, with care taken to prevent aggregation. The preparations resulted in a cell population of > 98% platelets. To obtain supernatant of the platelets, the suspension in DMEM was adjusted to 6.0×10^8 platelets/ml and the preparation was incubated at 37°C for 4 hr with 0.1 mM ADP in a CO_2 incubator (95% air-5% CO_2). After a 4-hr incubation, the suspension of platelets was centrifuged at 1700 × *g* for 15 min, filtered and the resulting supernatant was used for the following experiments.

Chemical activation of latent TGF-β1. Activation of latent TGF-β1 by acid and heat treatment was done, as described elsewhere (Lawrence *et al.*, 1985; Piao *et al.*, 1990). Briefly, supernatant of platelets was acidified with sterile 5 mol/l of HCl (final pH, ~2.5), incubated at 37°C for 30 min and neutralized with sterile 4 mol/l of NaOH. Heat activation was carried out by incubating the supernatant at 80°C for 10 min.

Cell culture. Porcine aortic ECs were cultured as described (Matsumura *et al.*, 1990). When ECs cultured in 12-well gelatin-coated plates became confluent, the culture medium was changed to supernatant with or without chemical treatments and was incubated for 12 hr at 37°C. In some experiments, TGF-β1 neutralizing antibody or control nonimmune IgG was added to the above supernatant at the beginning of incubation. At the end of incubation, the supernatant was aspirated off, boiled for 5 min and centrifuged at 8000 × *g* for 5 min. The resulting supernatant was then collected and stored at −20°C until assay for ET-1. For Northern blot analysis, ECs grown in 60-mm gelatin-coated Petri dishes were incubated with the supernatant described above for 2 ~~ 12 hr, these cells served as the sample for total RNA extraction.

RIA for determination of ET-1. RIA for ET-1 was performed as described previously (Ikegawa *et al.*, 1990). ET-1 antiserum (a gift from Dr. M. R. Brown, University of California, San Diego, CA) did not cross-react with big ET-1, as described by Hexum *et al.* (1990). By using reverse-phase high-performance liquid chromatography coupled with RIA, we characterized the immunoreactive ET in the culture medium obtained from ECs after the incubation. The elution profiles revealed one major immunoreactive ET component at the elution position corresponding to synthetic porcine ET-1.

Total RNA extraction and Northern blot analysis. After the incubation, total RNA extraction was done by using an LiCl-urea technique. Briefly, the cell monolayer was washed and lysed by direct exposure to 3 M LiCl/6 M urea. These cells were immediately scrapped off with a Cell Lifter (Costar, Cambridge, MA), sonicated and incubated overnight at 4°C. Subsequently, the cell lysates were centrifuged at 12,000 × *g* for 10 min, and the pellets were resuspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.5% SDS and 200 μg/ml of proteinase K. After incubation for 4 hr at 37°C, total RNA was purified by phenol/chloroform extraction and ethanol precipitation.

The isolated total RNA was then separated in formaldehyde-1.1% agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N^+, Amersham, Tokyo, Japan) in the presence of 20 × SSC (3 M sodium chloride and 0.3 M sodium citrate, pH 7.2). Subsequently, the nylon membrane was hybridized in hybridization buffer (6 × SSC, 0.01 M EDTA, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml of sheared, denatured salmon sperm DNA) at 67°C with porcine prepro ET-1 cDNA probe (a gift from Dr. K. Goto, University of Tsukuba, Tsukuba, Japan) and GAPDH cDNA probe (Clontech, Palo Alto, CA) labeled with [α-^{32}P]dCTP by using a Random Primer DNA Labeling Kit (Takara Shuzo, Kyoto, Japan). After hybridization, the membrane was washed twice in 2 ×, once in 1 × and twice in 0.1 × SSC containing 0.1% SDS, and autoradiography was then done with intensifying screens at −80°C by exposure to X-Omat AR film (Kodak) for 12 hr.

The autoradiograms of ET-1 were quantified by densitometric analyses, and the signals of ET-1 mRNA were normalized for each sample with respect to the density of the corresponding GAPDH mRNA signals.

Detection of active TGF-β1. Concentrations of active TGF-β1 in the supernatant of platelets were estimated by sandwich enzyme-linked immunosorbent assay by using a TGF-β1 enzyme-linked immunosorbent assay System Kit (Amersham). This assay system did not react to latent TGF-β1, and the detection limit of the assay was 0.1 ng/ml (4 pM). The cross-reactivity with TGF-β2 was below 0.6%. In some experiments, ECs were incubated with DMEM alone and the active TGF-β1 concentrations also were determined in a same manner.

Statistical analysis. All values are given as mean ± S.E.M.. Statistical analyses were performed by using analysis of variance followed by Dunnett's or Tukey's multiple comparison tests. Differences were considered significant at $P < .05$.

Chemicals. ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan). Adenosine 5'-diphosphate disodium salt was obtained from Kohjin Co., Ltd. (Tokyo, Japan). Human TGF-β1 and its neutralizing antibody were purchased from R&D systems (Minneapolis, MN). This antibody neutralizes the biological activities of TGF-β1, TGF-β2 and TGF-β3 (manufacturer's information). [^{125}I]ET-1 and [α-^{32}P]dCTP were purchased from Amersham Japan Ltd. (Tokyo, Japan). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Active TGF-β1 concentration in the supernatant. Cultured ECs secreted active TGF-β1 into the medium, but the amount of this molecule was below the detection limit (<4 pM) after incubation with DMEM alone for 12 hr (table 1). As shown in table 1, when the platelet suspension adjusted to 6.0×10^8 platelets/ml was incubated for 4 hr, the concentration of active TGF-β1 in the supernatant was 30.7 ± 2.8 pM. ADP (0.1 mM), at a dose which induces platelet aggregation (Slivka and Loskutoff, 1991), did not significantly change the release of active TGF-β1 from platelets. When chemical activation was done by transient acidification (pH 2.5 for 30 min) or heating (80°C for 10 min), the concentration of active TGF-β1 in the supernatant was increased markedly to 456.1 ± 5.0 and 289.8 ± 8.3 pM, respectively.

Effects of chemical activation on the supernatant-induced ET-1 release. As shown in figure 1, the basal

TABLE 1

Concentrations of active TGF-β1 in the supernatant

The supernatant was obtained by centrifugation of platelets after incubation with or without ADP (0.1 mM) for 4 hr, and chemical activation was then performed. For acid activation, the supernatant was treated with 5 N HCl for 30 min at 37°C and neutralized by 4 N NaOH. For heat activation, the supernatant was incubated at 80°C for 10 min. In some experiments, ECs were incubated with DMEM alone for 12 hr and active TGF-β1 concentration in the medium was determined. Each value represents the mean ± S.E.M. (n = 4).

Sample	Treatment	TGF-β1
		pM
EC conditioned medium	None	<4.00
Platelets (6.0×10^8 cells/ml)	None	30.72 ± 2.78
Platelets with ADP	None	41.09 ± 1.95
Platelets with ADP	pH 2.5	456.06 ± 5.03*
Platelets with ADP	80°C	289.84 ± 8.31*

* $P < .01$, compared with the supernatant prepared from platelets coincubated with ADP with no treatment.

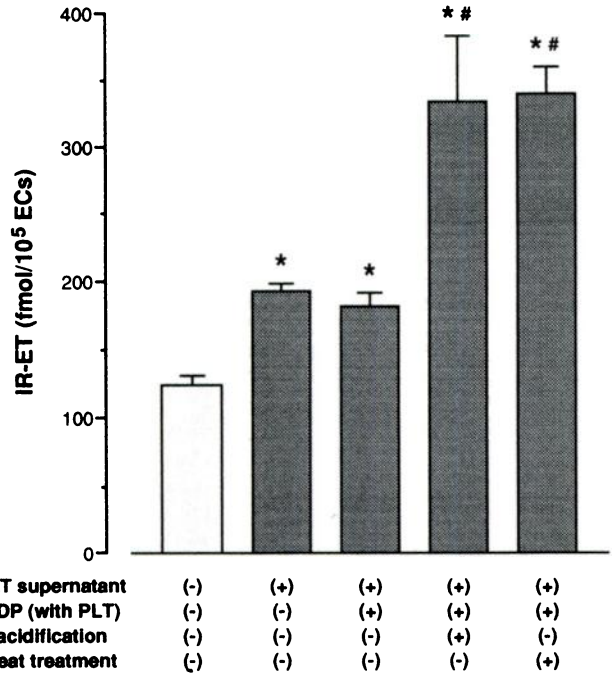


Fig. 1. Effects of chemical activation on platelet (PLT) supernatant-induced ET-1 release from cultured porcine aortic ECs. The ECs were incubated for 12 hr in the presence of PLT supernatant, with or without 0.1 mM ADP. Chemical activation of latent TGF-β1 was done as described in table 1. Each column and bar represents the mean ± S.E.M. (n = 4~13). *$P < .01$, compared with the basal value. #$P < .001$, compared with supernatant without chemical treatments. IR, immunoreactive.

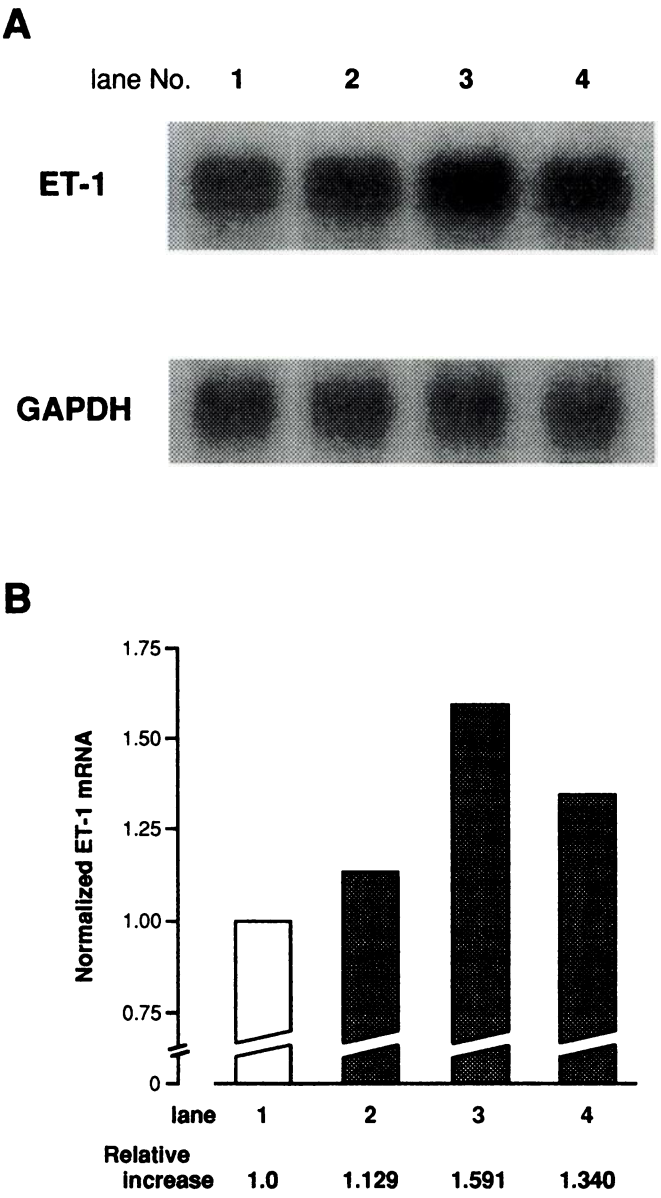
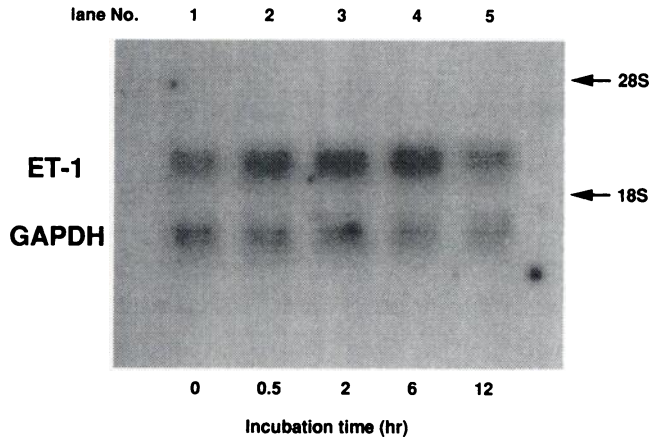


Fig. 2. Northern blot analysis of prepro ET-1 mRNA in cultured porcine aortic ECs. Total RNA (10 μg/lane) was hybridized with porcine prepro ET-1 and GAPDH cDNA as probes. ECs were incubated with supernatant of platelets activated by ADP for 2 hr, with or without additional chemical activation. The signals for GAPDH mRNA for each lane are shown as internal control (A). The signals for ET-1 mRNA were normalized to the corresponding GAPDH signals, quantified by densitometric analyses and the relative increases in normalized ET-1 mRNA are shown (B). Lane 1, control; lane 2, platelet supernatant stimulated by ADP (supernatant); lane 3, acidified supernatant; and lane 4, heated supernatant. Each column represents the mean of two experiments.

release of ET-1 from ECs during a 12-hr incubation was 125 ± 7 fmol/10^5 ECs. When ECs were incubated with the supernatant prepared from platelets alone, ET-1 release from the cells was increased significantly to 194 ± 6 fmol/10^5 ECs from the basal value. Similar increment was observed with platelet supernatant after ADP treatment. When the supernatant was exposed to acidification and heat treatment, ET-1 release was increased markedly to 335 ± 19 and 341 ± 19 fmol/10^5 ECs, respectively.

Effects of chemical activation on the supernatant-induced stimulation of ET-1 gene expression. Figure 2 shows the typical pattern of endothelial prepro ET-1 mRNA expression (fig. 2A) and the relative increases of ET-1 mRNA, normalized by corresponding GAPDH signals, from basal values (fig. 2B). Prepro ET-1 mRNA expression was increased slightly in the presence of the supernatant without chemical activation (fig. 2, lane 2) from ECs alone (fig. 2, lane 1, control). When the supernatant was acidified (fig. 2, lane 3), the expression of ET-1 mRNA normalized by GAPDH signal was increased markedly to 1.6-fold of the control. Heat-activated supernatant (fig. 2, lane 4) was less potent (1.3-fold increase) compared with acid activation.

Time-dependent effect of heat-activated supernatant on ET-1 gene expression. As shown in figure 3, when



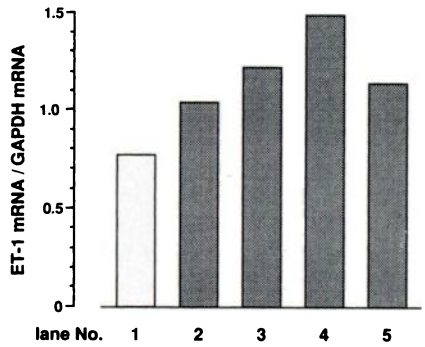


Fig. 3. Time course of prepro ET-1 mRNA induction by heat-activated supernatant. ECs were incubated for the indicated times in the presence of heat-activated supernatant. Details are similar to those shown in figure 2.

ECs were exposed to heat-activated supernatant for indicated times, prepro ET-1 mRNA expression was increased and reached to maximum at 6 hr after the addition of the supernatant (fig. 3, lane 3). After this, the expression of ET-1 mRNA returned to basal level (fig. 3, lane 1) by the 12-hr incubation (fig. 3, lane 5).

Dose-dependent effect of TGF-β1 on ET-1 release from ECs. As shown in figure 4, when ECs were incubated with various concentrations of purified human TGF-β1 for 12 hr, ET-1 release was increased in a dose-dependent fashion. The augmentation of ET-1 release was statistically significant (≥8 pM), and the maximal stimulation was observed at 200 pM, a value about 1.8-fold over basal values. At higher concentrations of TGF-β1 (over 200 pM), ET-1 release was unexpectedly reduced, but differences in ET-1 levels were still significant over controls.

Relationship between TGF-β1 concentration and ET-1 production. Figure 5 shows the different profiles of supernatant- and TGF-β1-induced stimulation of endothelial ET-1 release. When ECs were exposed to the supernatant with or without chemical activation, ET-1 production was enhanced in a TGF-β1 concentration-dependent manner. On the other hand, when purified TGF-β1 was added to ECs at concentrations which were similar to those in the platelet supernatant after chemical activation, the agent-induced enhancement of ET-1 release was declined, as stated above.

Effects of TGF-β1 neutralizing antibody on chemically activated supernatant-induced ET-1 production.

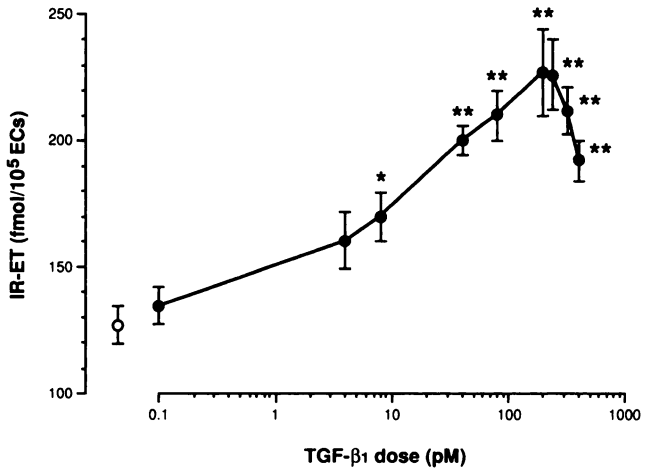


Fig. 4. Effects of TGF-β1 on ET-1 release from cultured ECs. The ECs were exposed to increasing concentrations of purified human TGF-β1 for 12 hr. Each point and bar represents the mean ± S.E.M. (n = 6). *P < .05, **P < .01, compared with the control value. ○, control; ●, TGF-β1 treated. IR, immunoreactive.

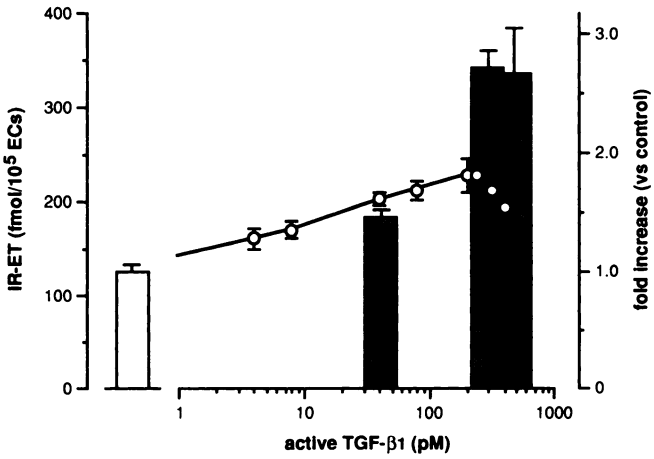


Fig. 5. Relationship between TGF-β1 concentration and ET-1 production. Released ET-1 levels and correlated TGF-β1 concentrations are summarized. The ECs were exposed to purified human TGF-β1 or supernatant of platelets, with or without chemical activation for 12 hr. Each point (column) and bar represents the mean ± S.E.M. (n = 4~13). Open bar, control; hatched bar, supernatant; ○, TGF-β1 treated. IR, immunoreactive.

In previous studies we found that the platelet supernatant-induced enhancement of ET-1 release was inhibited significantly by TGF-β1 neutralizing antibody, but not by control nonimmune IgG (Matsumura *et al.*, 1994: Umekawa *et al.*, 1994). Although this antibody (5 μg/ml) efficiently suppressed the supernatant-induced action, the corresponding TGF-β1 concentration in the supernatant was only 30 pM. When we used chemically activated supernatant, the amount of TGF-β1 was over 300 pM (table 1). To determine the importance of TGF-β1 in the supernatant-induced action, we examined the effect of TGF-β1 neutralizing antibody on the chemically activated supernatant-induced ET-1 release. This antibody neutralizes the biological activities of TGF-β1, TGF-β2 and TGF-β3 (manufacturer's information). First, we defined the dosage of the antibody that completely inhibited maximum stimulation on ET-1 release by 200 pM TGF-β1. As shown in figure 6, TGF-β1-induced maximal stimulation was completely inhibited by 15 μg/ml of antibody. Figure 7

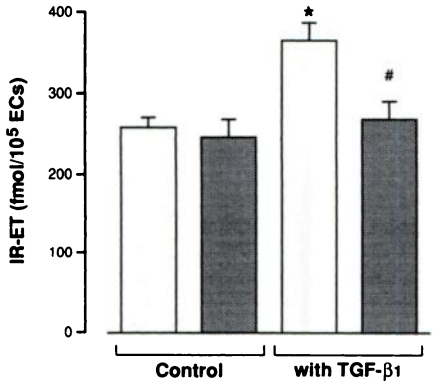


Fig. 6. Inhibitory effects of TGF-β1 neutralizing antibody on TGF-β1-induced endothelial ET-1 production. The ECs incubated with 200 pM TGF-β1 for 12 hr were supplemented with TGF-β1 neutralizing antibody or control nonimmune IgG (15 μg/ml) at the beginning of incubation. Each column and bar represents the mean ± S.E.M. (n = 7). *P < .01, compared with the corresponding control. #P < .01, compared with IgG treatment. Open bar, IgG; hatched bar, TGF-β1 neutralizing antibody treated. IR, immunoreactive.

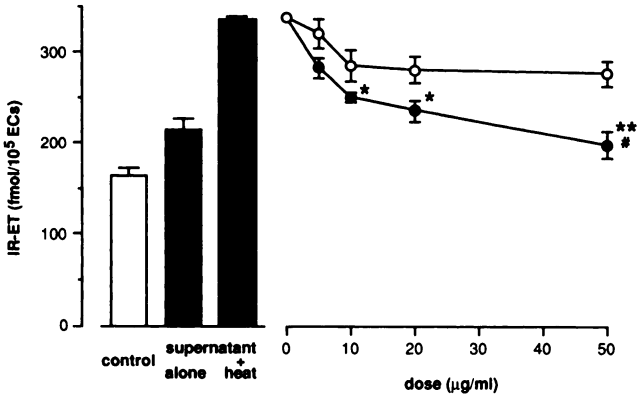


Fig. 7. Dose-dependent effects of IgG and TGF-β1 neutralizing antibody on heat-activated supernatant-induced endothelial ET-1 production. The ECs were exposed to supernatant with or without the indicated doses of IgG and TGF-β1 neutralizing antibody for 12 hr. Each column (point) and bar represents the mean ± S.E.M. (n = 4~6). *P < .01, **P < .001, compared with the supernatant with heat activation. #P < .01, compared with same dose of IgG treatment. ○, Ig G; ●, TGF-β1 neutralizing antibody treated. IR, immunoreactive.

shows dose-dependent effects of TGF-β1 neutralizing antibody and control nonimmune IgG on heat-activated supernatant-induced stimulation of ET-1 release. Although control IgG decreased the heat-activated supernatant-induced ET-1 release, the suppressive effects were not statistically significant. In contrast, TGF-β1 neutralizing antibody dose-dependently decreased the heat-activated supernatant-induced ET-1 release, and the suppressive effects were statistically significant, at over 10 μg/ml. Moreover, this antibody at 50 μg/ml dosage significantly reduced the supernatant-induced ET-1 release, compared with the case of the same dose of IgG.

Discussion

We obtained evidence that platelets spontaneously secrete active TGF-β1, but secretion was not influenced when platelets were activated and/or aggregated by ADP treatment. However, the active TGF-β1 concentration in the supernatant was greatly increased by treatments such as transient acidification and heating: these treatments are known to activate the latent TGF-β1 (Lawrence *et al.*, 1985; Piao *et al.*, 1990). Thus, platelet-derived TGF-β1 released in response to ADP stimulation was mostly biologically inactive latent form. This finding is in agreement with the results obtained by Miyazono *et al.* (1988) and Slivka and Loskutoff (1991).

It is well known that aggregated and/or activated platelets release various bioactive substances, including TGF-β1. We have found that supernatant-induced ET-1 release was potentiated when platelets were aggregated by thrombin (Matsumura *et al.*, 1994). However, our results showed that ADP-induced platelet aggregation did not induce further potentiation of supernatant-induced ET-1 production. These results support the above interpretation that TGF-β1 is released in an almost latent form during ADP-induced platelet aggregation, and also suggest that platelet-induced stimulation of endothelial ET-1 production did not merely depend on activation and/or aggregation.

In the present study, we found that acidification or heat treatment of platelet supernatant obtained after activation by ADP enhanced the supernatant-induced stimulation of ET-1 production. The above treatments are known to change the biologically inactive latent TGF-β1 to an active form (Lawrence *et al.*, 1985; Piao *et al.*, 1990). In fact, we noted marked increases in active TGF-β1 concentrations in the supernatant after acidification or heat treatment. These findings strongly support our recent proposal that TGF-β1 is responsible for the platelets-induced enhancement of ET-1 production (Matsumura *et al.*, 1994).

Several agents such as angiotensin II, arginine-vasopressin and TGF-β1 stimulate ET-1 synthesis at both message and protein levels (Kurihara *et al.*, 1989; Emori *et al.*, 1991). To determine if enhancement of the supernatant-induced ET-1 release was due to stimulation of gene expression, effects of supernatant with or without acidification or heat treatment on endothelial prepro ET-1 mRNA expression were examined by using Northern blots. Compared with the supernatant alone, acidified or heated supernatant stimulated the expression of ET-1 message. We also examined the time course of the expression of prepro ET-1 mRNA stimulated by heat-activated supernatant. ET-1 mRNA levels were gradually increased for several hours, followed by the restoration to basal levels. A similar profile of ET-1 gene expression was observed when ECs were exposed to TGF-β1 (Kurihara *et al.*, 1989). Thus, it is likely that acidification and heat treatment of the platelet supernatant activates latent TGF-β1 released during platelet aggregation and that activated TGF-β1 enhances endothelial ET-1 production, at the message level.

To evaluate the importance of TGF-β1 in the supernatant-induced action, we first determined the dose-dependency of TGF-β1 on endothelial ET-1 production. Purified human TGF-β1 (0.1 ~ 400 pM) dose-dependently increased ET-1 release from ECs, but these increments declined at higher concentrations, the reason for this being unclear. Suga *et al.* (1992) reported that TGF-β1 was a powerful stimulator of C-type natriuretic peptide release from ECs, at both the protein and message levels. According to their report, TGF-β1 dose-dependently stimulates the release of natriuretic peptide and the potency of this peptide in stimulating natriuretic peptide production was greater than that of ET-1 production. On the other hand, Kohno *et al.* (1993) reported

that other types of natriuretic peptides inhibit the release of ET-1 from cultured cells. In addition, other investigators demonstrated that the production of ET-1 was regulated by inhibitory stimuli such as nitric oxide and prostacyclin (Boulanger and Lüscher, 1990; Yokokawa *et al.*, 1991). Under such conditions, these factors may contribute to the negative regulation of ET-1 production when the biosynthetic pathway is upregulated.

In contrast to ET-1 release in response to the purified TGF-β1, when ECs were exposed to various conditions of platelet supernatant, endothelial ET-1 production was enhanced, in a TGF-β1 concentration-dependent manner, and no suppressive effects were observed even at doses with which purified TGF-β1-induced actions declined. In addition, the supernatant-induced action was much more efficacious than the TGF-β1-induced maximal effect. In the case of the supernatant, platelet-derived TGF-β1 might interact with other simultaneously released platelet-derived factors, and these interactions may result in a additive or synergistic potentiation on ET-1 production. Our results showed that TGF-β1 neutralizing antibody significantly inhibited the heated supernatant-induced potentiation on ET-1 release, but the suppression was incomplete. The results suggest that the supernatant (after chemical activation of latent TGF-β1)-induced stimulation of endothelial ET-1 production is due mainly to the release and activation of TGF-β1 from platelets. But, simultaneously released TGF-β1 may further stimulate the ET-1 synthesis by interacting with other platelet-derived factors, as suggested previously by Ohlstein *et al.* (1991).

TGF-β belongs to a family of 25 kD homodimeric proteins and the existence of three isoforms (TGF-β1, TGF-β2 and TGF-β3) are known in mammalian tissues. In general, all three isoforms have similar structural features and biological activities, but tissue distributions of these isoforms differed: TGF-β1 and TGF-β2 predominantly exist in platelets and bone, but TGF-β3 is found mainly in cells of mesenchymal origin (Roberts and Sporn, 1990; Segarini, 1993). Cheifetz *et al.* (1990) reported that the action of TGF-β2 are at least 50-fold less potent than that of TGF-β1 and TGF-β3 in certain cells, particularly ECs. Taken together, we considered that TGF-β1 was a major component of the isoforms which mediate platelet-induced stimulation of endothelial ET-1 production. We cannot rule out the contribution of other platelet-derived TGF-β isoforms on platelet-induced ET-1 synthesis.

TGF-β1 neutralizing antibody significantly inhibited the heated supernatant-induced potentiation of ET-1 production. However, the reduction of supernatant-induced action also was observed when control nonimmune IgG was used, but the changes were not statistically significant. Previously, we observed similar IgG-induced suppression on supernatant-induced ET-1 production (Umekawa *et al.*, 1994). These effects may be caused by immunologic or interspecies interaction between ECs and platelets, because ECs and platelets were prepared from the same species (bovine) and no IgG-induced suppression was observed in such experiments (Matsumura *et al.*, 1994).

It has been reported that platelets secrete various bioactive substances such as serotonin, ADP, cyclooxygenase products and numerous growth factors. Studies indicated that these factors did not influence endothelial ET-1 production (Kurihara *et al.*, 1989; Ohlstein *et al.*, 1989, 1991). On the other hand, Kohno *et al.* (1993) reported that PDGF stimulates ET-1 secretion from cultured rat mesangial cells, and others reported that TGF-β1 potentiates PDGF-induced action *via* the induction of its *beta* receptor gene expression (Janat and Liau, 1992). Therefore, PDGF may be a candidate for the potentiator of TGF-β1-induced stimulation on endothelial ET-1 production.

Sato *et al.* (1993) reported that latent TGF-β1 was activated during coculture of ECs and smooth muscle cells, and that targeting of latent TGF-β1 to smooth muscle cells was required for the activation mediated by plasmin. To date, plasmin is the most plausible candidate for the physiological activator of latent TGF-β1. This coculture system may be extrapolated to the case of atherosclerosis. Indeed, TGF-β1 and PDGF mRNA expression was increased in regions of experimentally induced atherosclerosis (Ross *et al.*, 1990). Moreover, others reported that the augmented release of ET-1 and its gene expression were observed in human atherosclerosis (Lerman *et al.*, 1991; Winkles *et al.*, 1993). In general, a large number of growth factors, cytokines and vasoregulatory molecules participate in pathogenesis of atherosclerosis (Ross, 1993). Under such conditions, activation of latent TGF-β1 released from platelets and its interaction with other factors may participate in the occurrence and/or progression of diseases through the release of ET-1. Although further investigation is required for the clarification of the involvement of platelet-induced enhancement on ET-1 production in pathological states, the present study suggests that platelet-induced ET-1 production closely depends on the amount of active TGF-β1.

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Send reprint requests to: Shiro Morimoto, Ph.D., Department of Pharmacology, Osaka University of Pharmaceutical Sciences, 2–10-65 Kawai, Matsubara, Osaka 580, Japan.