Effect of Growth Factors on Human Vascular Endothelial Cell Prostacyclin Production

Ari Ristimäki, Olavi Ylikorkala, and Lasse Viinikka

Prostacyclin (PGI₂) is an antithrombotic factor, which may prevent the initiation and the complications of arteriosclerosis. The most important site of PGI₂ production is the vascular endothelium, but little is known about how this process is regulated. In this connection, there is special interest in the roles of various growth factors released from platelets, macrophages, vascular smooth muscle cells, and the endothelial cells themselves. We investigated the effects of transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and acidic and basic fibroblast growth factors (aFGF and bFGF) on the PGI₂ production of cultured human umbilical vein endothelial cells by measuring the stable metabolite of PGI₂, 6-keto-prostaglandin F₁α, by radioimmunoassay. TGF-β induced dose- and time-dependent stimulation of PGI₂ production. The lowest stimulatory concentration of TGF-β was 0.1 ng/ml, and the maximal response, a 2.1-fold rise, was obtained with 1.0 ng/ml. The effect of TGF-β lasted 48 hours and was blocked by inhibitors of transcription, translation, and cyclooxygenase. Maximal stimulation by TGF-β was enhanced by epidermal growth factor. PDGF and bFGF had no effect on PGI₂ production, but aFGF inhibited it. This is the first demonstration that TGF-β enhances PGI₂ production by human vascular cells, and this phenomenon may be part of negative feedback mechanisms that prevent thrombosis and arteriosclerosis.

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Prostacyclin (PGI₂), a major arachidonic acid metabolite of vascular endothelial cells, is a powerful vasodilator and a potent inhibitor of platelet aggregation. It also inhibits the growth of human vascular smooth muscle cells in vivo, possibly by preventing the release of mitogens for these cells from macrophages, platelets, and endothelial cells, and by directly inhibiting smooth muscle DNA synthesis. Further, PGI₂ stimulates the fibrinolytic activity in atherosclerotic patients and may inhibit foam cell formation and cholesterol accumulation in the vascular wall. Thus, PGI₂ is an important antithrombotic and antiatherogenic factor, but little is known about the regulation of its production.

According to the original response-to-injury model, injury of the vascular wall and subsequent denudation of the endothelium caused platelet adhesion, aggregation, and the release of platelet growth factors, which were responsible for the initiation of atherosclerosis. Now it is known that, in addition, growth factors derived from macrophages, smooth muscle, and endothelial cells may be involved. These growth factors include platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor-α and factor-β (TGF-α and TGF-β).

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Recently, we showed that EGF, which is released from human platelets during aggregation, and TGF-α, another ligand of the same receptor, stimulate the in vitro production of PGI₂ in human umbilical vein endothelial cells. Thus these factors could prevent thrombosis and atherogenesis. To clarify further the role of growth factors, we studied the effects of TGF-β, PDGF, acidic FGF (aFGF), and basic FGF (bFGF) on PGI₂ synthesis by human umbilical vein endothelial cells.

Methods

Materials

Medium 199 and fetal calf serum were obtained from Flow Laboratories, Ayshire, U.K. Penicillin, streptomycin, and L-glutamine were purchased from Gibco, Grand Island, NY. Collagenase CLS 3 was obtained from Worthington Biochemicals, Freehold, NJ. Gelatin was purchased from Merck, Darmstadt, F.R.G. Cycloheximide, actinomycin D, and mouse EGF were obtained from Sigma Chemical, St. Louis, MO. Indomethacin was purchased from Denux, Copenhagen, Denmark. Porcine TGF-β1 was obtained from R&D Systems, Minneapolis, MN. PDGF and bFGF were purchased from AMGen, Thousand Oaks, CA. aFGF was obtained from Collaborative Research, Bedford, MA. All culture flasks and plates were purchased from Nunc, Roskilde, Denmark.

Endothelial Cell Cultures

Endothelial cell cultures were prepared from human umbilical cord veins with the approval of the local committee of ethics and according to the method originally described by Jaffe et al. with slight modifications.
Incubation Procedures

The effects of the growth factors on PGI2 production were studied in 96-well plates by using confluent monolayers of endothelial cells after the first passage. Immediately before each experiment, the culture medium (Medium 199 supplemented with 20% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) was aspirated from the confluent monolayers, and these were washed once with 0.25 ml of culture medium. Fresh culture medium (0.25 ml) was added without (control) or with 0.01 to 10 ng/ml of TGF-β or 0.1 to 100 ng/ml of PDGF, bFGF, aFGF, or EGF. Cultures were incubated for 1 to 72 hours at 37°C in 5% CO2 in air.

Cycloheximide (2 μg/ml), actinomycin D (2 μg/ml), and indomethacin (1.4 μM) were added simultaneously without (control) or with TGF-β (1.0 ng/ml) and incubated for 8 hours.

At the end of each incubation, the medium was placed in a polypropylene tube and was stored immediately at -20°C.

Analytical Methods

PGI2 synthesis was measured by radiomunounassay of 6-keto-prostaglandin F1α (6-keto-PGF1α), a stable metabolite of PGI2.

Statistical Analysis

Statistical significance was calculated using Student’s unpaired t test in the case of a single comparison. For multiple comparison, the t test was used only if one-way analysis of variance had shown a significant difference. All results are expressed as the means±SEM.

Results

TGF-β increased the release of 6-keto-PGF1α in a dose-dependent manner. The lowest stimulatory concentration was 0.1 ng/ml, and the concentration giving the maximal (2.13±0.10-fold) response was 1.0 ng/ml (Figure 1). The effect of TGF-β was time-dependent and increased progressively between 3 and 48 hours (Figure 2). After incubation for 3 hours, the increase was 1.47±0.17-fold (p<0.05) calculated from nine replicate determinations in three separate experiments, whereas incubation for 1 hour in the same experiments was ineffective. The maximal effect of TGF-β was enhanced by 1 ng/ml of EGF, which by itself caused a 1.40±0.17-fold and together with TGF-β, a 3.51±0.06-fold increase in the release of 6-keto-PGF1α (Figure 3).

PDGF, aFGF, and bFGF did not stimulate 6-keto-PGF1α release (Figure 1). In fact, aFGF was inhibitory at concentrations of 10 ng/ml (Figure 1) and 100 ng/ml (not shown). The two concentrations of aFGF were equally potent.

Actinomycin D and cycloheximide blocked the stimulatory effect of TGF-β, indicating that stimulation was dependent on de novo synthesis of RNA and protein, respectively (Table 1). Indomethacin also inhibited the stimulatory effect of TGF-β and decreased the basal production of 6-keto-PGF1α by 97%, confirming the key role of cyclooxygenase.

Discussion

TGF-β is a multifunctional peptide, which exerts either stimulatory or inhibitory effects on proliferation, differentiation, and other cell functions. TGF-β prevents the growth of human vascular endothelial cells in vitro.1,2 TGF-β is usually liberated from cultured cells as an inactive compound, but plasmin can activate it.8 TGF-β is angiogenic in mice in vivo.20 The explanation of this may be that it is an attractant for macrophages and may stimulate the release of angiogenic peptides from them. TGF-β enhances the synthesis of endothelin23 and plasminogen activator inhibitor type I,24 elevates the expression of PDGF,25 and inhibits the production of plasminogen activators in vascular endothelial cells.26 Human platelets contain large amounts of TGF-β,19 now known as TGF-β1,27 but macrophages and endothelial cells can also release it.6,11 TGF-β is usually liberated from cultured cells as an inactive compound, but plasmin can activate a small pool of this latent TGF-β.27 However, in cocultures of endothelial and smooth muscle cells, TGF-β is activated by plasmin, causing an inhibition of endothelial cell movement.25 Thus, sufficient amounts of active TGF-β may be present in the vascular wall and especially at a site of endothelial injury where platelets are aggregating to cause biologically important effects. The intracellular signal mechanisms underlying the multiple actions of TGF-β remain essentially unknown.20 Interestingly, it has been suggested that the antimitogenic effect of TGF-β is dependent on de novo synthesis of prostaglandins.20

The present data are the first demonstration that TGF-β stimulates the synthesis of PGI2 in any type of endothelial or...
Figure 2. Time course of transforming growth factor-β (TGF-β) stimulated 6-keto-prostaglandin F₁α (6-keto-PGF₁α) release. Human umbilical vein endothelial cells were incubated without (control) or with TGF-β (1.0 ng/ml) for the periods of time indicated. The values are means±SEM from three replicate determinations. The first statistically significant (p<0.05) difference in 6-keto-PGF₁α production between TGF-β treated cells and controls occurred after a 3-hour incubation.

Figure 3. Effect of adding transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) together. Human umbilical vein endothelial cells were incubated without (control) or with TGF-β (1.0 ng/ml) and/or EGF (1.0 ng/ml) for 24 hours. The values are the means±SEM of six replicate determinations in two separate experiments. The statistical significance was calculated against the control.

Table 1. Effect of Inhibitors on Transforming Growth Factor-β-Induced 6-Keto-Prostaglandin F₁α Release

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>6-keto-PGF₁α (ng/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.16±0.18</td>
</tr>
<tr>
<td>TGF-β</td>
<td>11.62±0.42*</td>
</tr>
<tr>
<td>Cycloheximide (2.0 μg/ml)</td>
<td>2.26±0.06*</td>
</tr>
<tr>
<td>Cycloheximide+TGF-β</td>
<td>2.21±0.15*</td>
</tr>
<tr>
<td>Actinomycin D (2.0 μg/ml)</td>
<td>8.18±0.85</td>
</tr>
<tr>
<td>Actinomycin D+TGF-β</td>
<td>7.12±0.24</td>
</tr>
<tr>
<td>Indomethacin (1.4 μM)</td>
<td>0.20±0.04*</td>
</tr>
<tr>
<td>Indomethacin+TGF-β</td>
<td>0.26±0.02*</td>
</tr>
</tbody>
</table>

The values are the means±SEM of three replicate determinations and are given in ng/ml. The experiment was repeated twice with similar results.

Confluent monolayers of human umbilical vein endothelial cells were incubated for 8 hours without (control) or with TGF-β (1.0 ng/ml) and/or with the indicated amounts of inhibitors. 6-keto-PGF₁α was measured by radioimmunoassay from conditioned medium.

*p<0.001 against the control.

PGF₁α=prostaglandin F₁α, TGF-β=transforming growth factor-β.

EGF potentiated the effect of TGF-β in the stimulation of endothelial cell PGF₁α production. A similar effect has been reported in cultured rat smooth muscle cells, in which TGF-β and EGF synergistically accelerated the recovery of PGF₁α synthesis after aspirin treatment, which is associated with increased production of cyclooxygenase mRNA. Unexpectedly, however, PGF₁α synthesis could not be inhibited by actinomycin D, but could be prevented by cycloheximide. Since TGF-β and EGF are both released from platelets, they are likely to act in concert at thrombotic sites. The combination of EGF and TGF-β may well be the platelet-derived stimulator of endothelial cell PGF₁α production implied by Tremoli et al. 27

aFGF and bFGF have an over 50% sequence homology and they act via the same high-affinity receptors that are also present in endothelial cells. They are potent angiogenic factors and induce all the basic mechanisms of angiogenesis, which include increased protease release from the endothelium, and migration, proliferation, and tube formation by the endothelial cells. bFGF is produced by macrophages and endothelial and smooth muscle cells, whereas aFGF is found mainly in neural tissue, but to some extent also in vascular smooth muscle cells. bFGF had no effect on endothelial PGF₁α synthesis, but aFGF inhibited it at the highest concentrations. This was somewhat unexpected, since bFGF is generally considered to be more potent than aFGF. However, aFGF was recently shown to inhibit the agonist-induced production of PGI₂ by human endothelial
cells, and thus our finding may represent a real difference between aFGF and bFGF, although no concentration dependence was found. On the other hand, aFGF and bFGF stimulated PG12 release by fetal bovine aortic endothelial cells and by human retinal capillaries, but these cells failed to respond to EGF, TGF-α, or TGF-β. It seems that the regulation of PG12 synthesis by growth factors is dependent on the species and on the type of vasculature from which the cells are originated.

PGDF is liberated not only from platelets, but also from macrophages, smooth muscle, and endothelial cells. Thus, PGDF must be present at the site of vascular injury. PGDF did not have any effect on endothelial cell PG12 production; this is compatible with the lack of PGDF receptors in large-vessel endothelial cells and in accordance with earlier data. The situation may be different in microvessel endothelial cells, which contain PGDF receptors and respond to PDGF. It remains to be discovered whether PGDF stimulates the production of PG12 by capillary endothelial cells.

In conclusion, TGF-β may act as an antiatherogenic compound by increasing the production of endothelial PG12, which prevents platelet aggregation, suppresses the proliferative capacity of smooth muscle cells, and decreases cholesterol accumulation in the vascular wall. The effect of TGF-β is potentiated by EGF.

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