Involvement of u-PA in the anti-apoptotic activity of TGFβ for vascular smooth muscle cells

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Abstract Previous studies suggest a role for the plasminogen or fibrinolytic system in the activation of latent-transforming growth β (L-TGFβ) into active TGFβ. In the present study, the anti-apoptotic activity of TGFβ on cultured vascular smooth muscle cells (SMC) isolated from the aorta of transgenic mice with single inactivation of genes encoding the tissue-type plasminogen activator (t-PA$^{-/-}$), urokinase-type plasminogen activator (u-PA$^{-/-}$), urokinase receptor (u-PAR$^{-/-}$) or plasminogen (Plg$^{-/-}$) genes was examined. Latent-TGFβ inhibited serum deprivation-induced apoptosis of SMC isolated from wild-type and t-PA$^{-/-}$ mice but failed to reduce apoptosis of SMC isolated from u-PA$^{-/-}$, u-PAR$^{-/-}$ or Plg$^{-/-}$ mice. Active TGFβ, however, was able to inhibit serum deprivation-induced apoptosis of these 5 cell types, indicating that u-PA and/or plasmin were involved in the activation of L-TGFβ. The anti-apoptotic effect of L-TGFβ could not be evoked by addition of exogenous t-PA to u-PA$^{-/-}$ cells, but was revealed by addition of exogenous u-PA or plasmin. This effect was dependent on the catalytic activity of plasmin as revealed by the dose-dependent inhibition of aprotinin or epsilon aminocaproic acid (EACA). These results therefore indicate that, at least in vitro, u-PA-mediated plasmin, through the generation of active TGFβ from L-TGFβ, is required for the anti-apoptotic activity of TGFβ on SMC.

Key words: u-PA; Smooth muscle cell; Tumor growth factor β; TGFβ; Apoptosis

1. Introduction

Apoptosis is a ubiquitous, evolutionarily conserved, physiological mechanism of cell death regulating tissue mass and cyto-architecture in many tissues [1]. Cell death is also prominent in human atherosclerotic plaques with areas of 'necrosis' being present in more than 80% of primary lesions [2–4]. Cell death has also been detected in animals models of atherosclerosis [5–7] and after balloon injury in animals [8,9]. The mechanisms triggering apoptosis in atherosclerotic lesions remain unknown. Several authors have proposed that growth factors and cytokines produced by macrophages and lymphocytes [10,11] contribute to apoptosis of vascular smooth muscle cells (SMC) during atherogenesis, a hypothesis that has been underscored by observations that some of these molecules promote apoptosis of SMC [4,12,13].

Transforming growth factor β (TGFβ) is a potent inhibitor of endothelial and smooth muscle cell proliferation, migration and protease synthesis [14–19]. It is secreted by multiple cell types in a latent pro-form (latent-TGFβ), from which mature TGFβ must be released in order to bind a cellular receptor and elicit a biological response [20,21]. Activation of latent-TGFβ in co-cultures of endothelial cells and SMC [22,23] is mediated by plasmin which cleaves of the aminoterminal propeptide from L-TGFβ [23–27]. Recently, evidence has been provided that binding of u-PA to the urokinase receptor (u-PAR) is required for plasmin-dependent conversion of latent-TGFβ [27]. This study and other recent investigations suggested that the fibrinolytic system may play an important role in cell proliferation and/or migration during atherosclerosis [28,29]. Indeed, several authors described both a mitogenic and a chemotactic effect of the tissue-type plasminogen activator (t-PA) and of u-PA for several cell types including vascular smooth muscle cells [30–34]. Notably, vascular SMCs express u-PA and t-PA during mitogenesis and migration in vitro and in vivo following vascular injury [32,35–37]. A role for u-PA in SMC migration in vascular wound healing and arterial neointima formation after injury was recently demonstrated by recent gene transfer and gene targeting studies in mice deficient for t-PA, u-PA, u-PAR and plasminogen (Plg) [28,34,38] which provided direct genetic evidence for a significant role of fibrinolytic enzymes in this process [28,34,38].

Because of this close interaction between TGFβ and the fibrinolytic system, this study was designed to investigate the activation of L-TGFβ and its apoptotic effect on aortic SMC isolated from mice deficient in t-PA, u-PA, plasminogen and u-PAR.

2. Materials and methods

2.1. Animals

Homozygous t-PA$^{-/-}$, u-PA$^{-/-}$, Plg$^{-/-}$ and u-PAR$^{-/-}$ mice were obtained and characterized as described previously [28,39,40].

2.2. Isolation and culture of SMC

SMC were isolated as described previously [41]. Briefly, media fragments of the aorta were incubated for 16 h at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 0.15% collagenase, 5% fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and glutamine (4 mM). After incubation, SMCs were sedimented by gentle centrifugation (400×*g*; 10 min), resuspended in DMEM+10% FCS and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium (DMEM+10% FCS) was changed every 3 days and a confluent SMC monolayer was obtained after about 7 days. Cells were routinely used from the third to the sixth passage.

2.3. Expression of TGFβ by SMC

Levels of active TGFβ in the conditioned media were determined with a specific ELISA (R&D, Abingdon, UK). Under these experimental conditions, the assay did not detect latent-TGFβ.

2.4. Measurement of apoptosis

SMC were seeded in 35 mm Petri dishes (5×10^5 cells/well) in DMEM+10% FCS and grown to confluence for 3 days. Culture me-

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dium was then aspirated, cells were rinsed and fresh medium+0.1% FCS was added in the presence of saline, latent-TGFβ, TGFβ (R&D, Abingdon, UK), plasmin, u-PA, t-PA, or plasmin inhibitors (epsilon aminocaproic acid: EACA or aprotinin) (Sigma Chemical Co., St. Louis, MO). Twenty-four hours later, apoptosis was measured with a photometric enzyme immunoassay (cell detection ELISA, Boehringer Mannheim, Mannheim, Germany) for the quantitative detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Apoptosis was expressed as mean number of oligonucleosomes/10^5 cells ± S.D. Results were from 3 different experiments performed in triplicate.

3. Results

When added to growth-arrested aortic SMCs isolated from wild-type, t-PA$^{-/-}$, u-PA$^{-/-}$, u-PAR$^{-/-}$ or plasminogen$^{-/-}$ mice, FCS depletion resulted in a dose-dependent pro-apoptotic effect (Fig. 1). As shown in Fig. 2A, the addition of active TGFβ to wild-type or any mutant SMC dose-dependently inhibited apoptotic cell death induced by FCS depletion with an IC_{50} value (dose which inhibited 50% of the pro-apoptotic activity of 0.1% FCS) around 1 ng/ml. When added to growth-arrested aortic SMCs isolated from wild-type mice or t-PA$^{-/-}$ mice, L-TGFβ also exhibited a dose-dependent anti-apoptotic activity (Fig. 2B). In contrast, L-TGFβ failed to reduce apoptotic cell death when added to SMC isolated from u-PA$^{-/-}$, u-PAR$^{-/-}$ or plasminogen$^{-/-}$ mice (Fig. 2B). These results indicate that both plasmin and u-PA are important for activation of L-TGFβ.

The importance of plasmin and u-PA in the activation of L-TGFβ was further emphasized by the observation that addition of u-PA to u-PA$^{-/-}$ SMC diminished the pro-apoptotic activity of 0.1% FCS (Fig. 3). The inhibitory activity of L-TGFβ also occurred when plasmin but not exogenous t-PA was added. The dose-dependent inhibitory effect of plasmin inhibitors (EACA and aprotinin) confirmed that plasmin

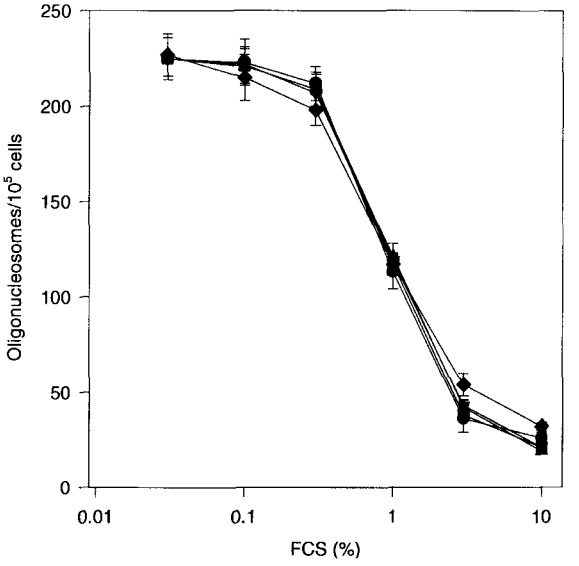

Fig. 1. Serum-induced apoptosis of SMCs isolated from transgenic mice. Confluent monolayers of SMCs isolated from wild-type (●), t-PA$^{-/-}$ (■), u-PA$^{-/-}$ (▲), u-PAR$^{-/-}$ (▼) or Plg$^{-/-}$ (◆) mice, cultured in 35 mm dishes were incubated for 24 h at 37°C in DMEM containing increasing concentrations of FCS. Apoptosis was measured with an ELISA kit as described under Section 2 and expressed as mean numbers of oligonucleosomes/10^5 cells ± S.D. (n = 6).

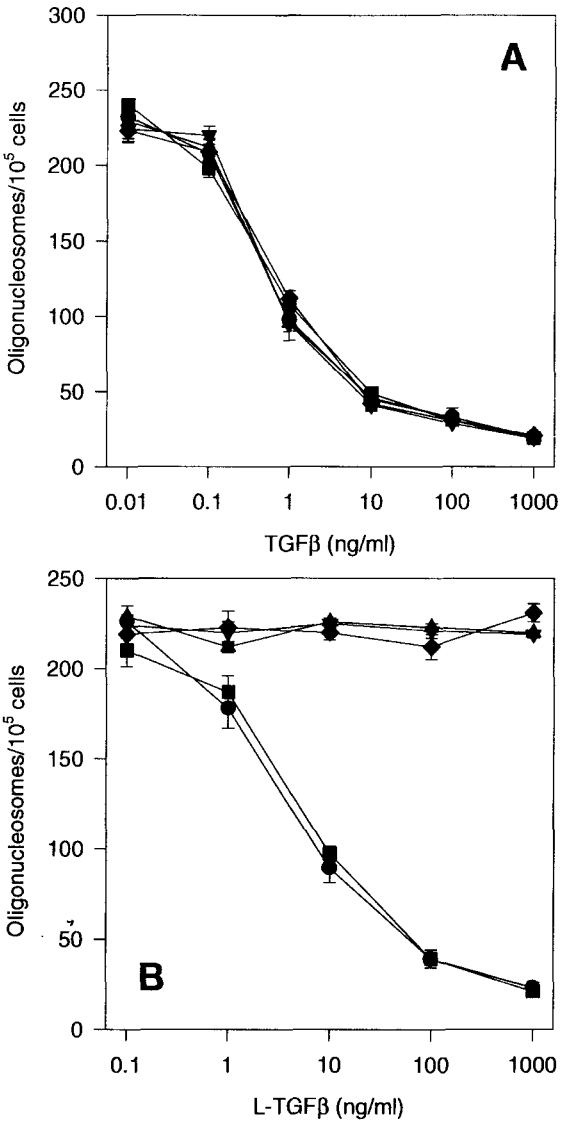

Fig. 2. Effect of L-TGFβ or TGFβ on serum-induced apoptosis of SMCs. Confluent monolayers of SMCs isolated from wild-type (●), t-PA$^{-/-}$ (■), u-PA$^{-/-}$ (▲), u-PAR$^{-/-}$ (▼) or Plg$^{-/-}$ (◆) mice, cultured in 35 mm dishes were incubated for 24 h at 37°C in DMEM containing 0.1% FCS and increasing concentrations of TGFβ (A) or L-TGFβ (B). Apoptosis was measured with an ELISA kit as described under Section 2 and expressed as mean numbers of oligonucleosomes/10^5 cells ± S.D. (n = 6).

mediated the conversion of L-TGFβ into active-TGFβ (Fig. 4). Moreover, the plasmin inhibitors were only effective when L-TGFβ but not TGFβ was added to the culture medium (Fig. 4). Quantitative determination of active TGFβ revealed that u-PA$^{-/-}$ SMC were only able to convert L-TGFβ to active TGFβ when catalytically-active u-PA or plasmin were added, since both EACA and aprotinin blocked this conversion (Fig. 5).

4. Discussion

Although vascular SMC accumulation is considered a key event in atherogenesis, specific regulatory mechanisms for cell proliferation and migration in the arterial wall are still poorly characterized. Cell death in human atherectomy and endarter-

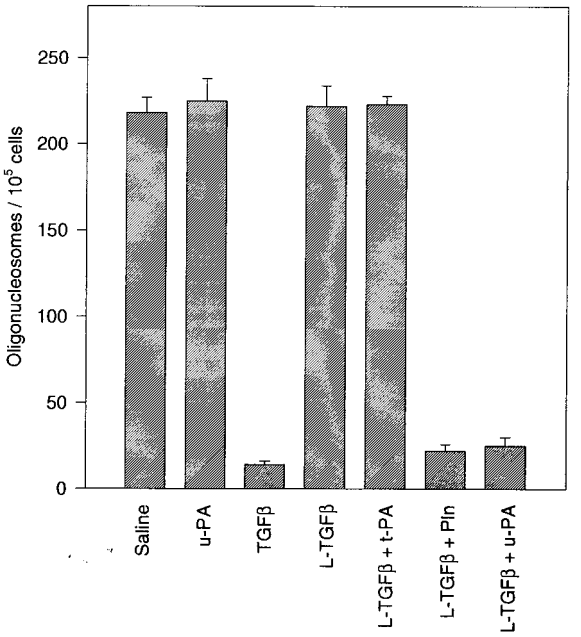

Fig. 3. Effect of u-PA on the apoptotic activity of FCS. Confluent SMCs from u-PA$^{-/-}$ mice were cultured for 24 h in DMEM+FCS (0.1%) in the presence of saline, murine u-PA (100 ng/ml), TGFβ (100 nM), L-TGFβ (100 nM) alone or associated with t-PA (100 ng/ml), plasmin (100 ng/ml) or u-PA (100 ng/ml). Apoptosis was then measured with an ELISA kit as described under Section 2 and expressed as mean numbers of oligonucleosomes/10^5 cells ± S.D. ($n = 6$).

ectomy specimens [2–4] and in animal models of vascular injury [5–7], may be a key feature that influences the size and stability of vessel wall lesions.

In vitro studies have identified several growth factors and cytokines that regulate apoptosis of vascular SMCs. Along with platelet derived growth factor-BB and insulin growth factor-1, several cytokines such as interferon-γ, tumor necrosis-α and interleukin-1β are now considered to be likely candidates [4,13].

Although TGFβ has opposite effects on proliferation versus migration of SMC [16,17,42,43], the precise molecular mechanisms for this dual role remain largely undefined. These different activities of TGFβ have been tentatively attributed to a decrease of cell-mediated proteolysis and the subsequent deposition of a new basement membrane and formation of junctional complexes between cells [16,18].

The present study demonstrates that TGFβ may result in SMC accumulation via a potent inhibitory effect on SMC apoptosis. Although the precise mechanism needs to be further defined, this effect may relate to the ability of TGFβ to increase intracellular cAMP levels [42]. Indeed, several studies have indicated a link between decreased apoptosis and increased intracellular cAMP levels [4].

A significant role by the fibrinolytic system in the development of arterial neointima formation and atherosclerotic lesions has been recently suggested by targeted gene manipulation of t-PA, u-PA, PAI-1, u-PAR and plasminogen [28,34,38,44]. Moreover, in a recent study, using SMC isolated from these transgenic animals, we provided direct evidence that u-PA and t-PA are implicated in SMC migration and growth induced by bFGF and PDGF-BB, respectively [45].

It has been demonstrated that plasmin may activate latent-TGFβ [24] in particular when u-PA was bound to its cellular receptor [27,43]. Our data that u-PA-deficient SMC were unable to convert latent-TGFβ into active TGFβ confirm and extend these observations. Moreover, u-PA, through its ability to generate plasmin, tightly controls the generation of active TGFβ. As such, activation of TGFβ and secondarily induced

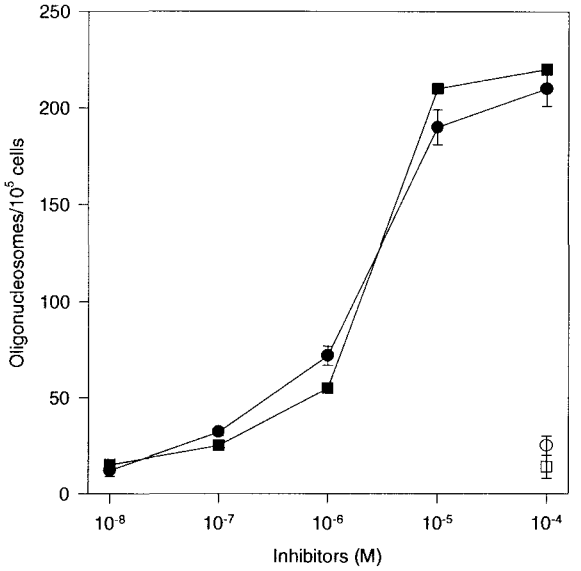

Fig. 4. Effect of plasmin inhibitors on the anti-apoptotic effect of L-TGFβ. Confluent SMCs from wild-type mice were cultured for 24 h in DMEM+FCS (0.1%) in the presence of L-TGFβ (100 ng/ml) (full symbols) or TGFβ (100 ng/ml) (empty symbols) and increasing concentrations of EACA (squares) or aprotinin (circles). Apoptosis was then measured with an ELISA kit as described under Section 2 and expressed as mean numbers of oligonucleosomes/10^5 cells ± S.D. ($n = 6$).

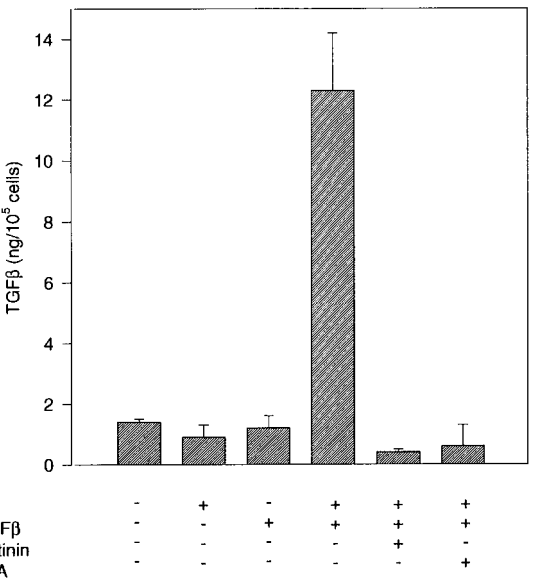

Fig. 5. Expression of TGFβ by SMC. Confluent SMC from u-PA$^{-/-}$ mice were incubated in DMEM+0.5% FCS in the presence of murine u-PA (100 ng/ml), L-TGFβ (100 ng/ml), aprotinin (10 μM) or EACA (10 μM) as indicated. After 24 h, the amount of TGFβ present in the culture medium was quantified as described under Section 2. Results are expressed as mean ± S.D. ($n = 6$).

expression of PAI-1 might contribute to a negative feedback loop in which increased u-PA expression by bFGF is counteracted by increased PAI-1 expression.

Our studies demonstrate an important modulatory role for u-PAR in the anti-apoptotic activity of L-TGFβ. SMC isolated from u-PAR-deficient mice did not respond to L-TGFβ and antibodies to u-PAR, as well as aprotinin and EACA, were also able to inhibit the anti-apoptotic activity of L-TGFβ (not shown), suggesting that u-PAR may modulate the anti-apoptotic activity of L-TGFβ in a plasmin-independent fashion. In our study, PAI-1 and α1-antitrypsin also showed an effect on the inhibitory effect of L-TGFβ with regard to the pro-apoptotic activity of 0.1% FCS (not shown) therefore confirming that, even when bound on its cell surface receptor, u-PA needs to be catalytically active to generate active TGFβ from L-TGFβ.

These results therefore indicate that, at least in vitro, u-PA-mediated plasmin, through its role in the generation of TGFβ from L-TGFβ is required for the control of the anti-apoptotic activity of TGFβ on SMC and provides direct genetic evidence for a causal role of u-PA in the regulation of the activity of TGFβ.

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