TGF-β-induced apoptosis in endothelial cells mediated by M6P/IGFII-R and mini-plasminogen

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Summary

Transforming growth factor-β (TGF-β), a key modulator of endothelial cell apoptosis, must be activated from the latent form (LTGF-β) to induce biological responses. In the present study, we report activation of TGF-β by functional and physical co-operation of the mannose-6-phosphate/insulin-like-growth-factor-II receptor (CD222) and the urokinase-type plasminogen activator receptor (CD87). We show that endothelial cells express CD222 and CD87 in a membrane complex and demonstrate that the association of these two receptors is essential for the release of active TGF-β in the transduced mouse fibroblast used as model cells. By contrast, smooth-muscle cells, which express CD222 and CD87 at similar density to endothelial cells but not in complexed form, do not activate TGF-β. We also have found that mini-plasminogen is a high-affinity ligand for CD222 and is essential for the activation of TGF-β by the CD87-CD222 complex to induce apoptosis in endothelial cells. This specific mechanism of TGF-β-mediated apoptosis in endothelial cells is thus a potential novel target to be considered for treatment of pathological vascular disorders (e.g. tumor angiogenesis).

Key words: Angiogenesis, Apoptosis, Fibrinolysis, TGF-β, Mini-plasminogen

Introduction

The balance between cell death and survival within blood vessels is decisive for the physiology of the vasculature. It also contributes to the pathogenesis of many diseases of the vascular system when dysregulated (Carmeliet, 2003). This control of vasculogenesis and angiogenesis is tightly coordinated by a network of cytokines, proteases, matrix proteins and other factors (Folkman and D'Amore, 1996; Jackson, 2002; Pepper, 2001; Risau, 1997). Transforming growth factor-β (TGF-β) was shown to play a key role during vascular remodeling by acting pleiotropically on the major cell types of the vasculature – endothelial cells (ECs), smooth-muscle cells (SMCs) and pericytes (Fajardo et al., 1996; Gajdusek et al., 1993; Hirschi et al., 1998; Iruela-Arispe and Sage, 1993; Koh et al., 1995; Madri et al., 1992; Plouet and Gospodarowicz, 1989; Roberts et al., 1986; Saksela et al., 1987; Sato et al., 1990; Sawdey et al., 1989; Vernon and Sage, 1999). At low doses, TGF-β contributed to angiogenesis, whereas, at higher concentrations, it both inhibited EC proliferation and migration, and stimulated SMC recruitment (Border et al., 1992; Carmeliet, 2003; Moses et al., 1990). Both proapoptotic influence on ECs and antiapoptotic influence on SMCs were ascribed to TGF-β (Pollman et al., 1999; Schulick et al., 1998). These data show that proper regulation of the amount of active TGF-β is essential for the function of the microvascular unit.

TGF-β is secreted as inactive complex, associated with latency-associated peptide (LAP) to form latent TGF-β (LTGF-β). Active TGF-β can be released from LTGF-β in vitro by low pH or heat (Brown et al., 1990). Several mechanisms have been proposed for LTGF-β activation in vivo (Crawford et al., 1998; Godar et al., 1999; Lyons et al., 1990; Munger et al., 1999). We hypothesize that differences between the individual cell types of the vasculature in their ability to activate LTGF-β could contribute to the pleiotropic, and often opposing, effects of TGF-β in vasculogenesis.

Previously, on the basis of in vitro experiments with purified proteins, we proposed a model for the activation of LTGF-β. In this model, the mannose-6-phosphate/insulin-like-growth-factor-II receptor (M6P/IGF2R, CD222) complexed LTGF-β, plasminogen and the urokinase-type plasminogen-activator receptor (uPA-R, CD87) (Godar et al., 1999) (for review, see Ghosh et al., 2003). We also found that CD222 was a receptor not only for LTGF-β but also for plasminogen (Plg), which was converted to plasmin (Plm) by CD87-bound urokinase. Based on TGF-β-activation assays on this in-vitro-assembled complex, we suggested that urokinase bound to CD87 converts

plasminogen bound to CD222 to plasmin in order to activate LTGF-β in living cells in vivo. The control over proteolysis of plasmin by this complex would regulate the release of active TGF-β from CD222-bound LTGF-β.

Prompted by this, we reconstituted the functional LTGF-β-activating complex in fibroblasts, and tested the role of TGF-β activation in ECs and SMCs. Although ECs and SMCs express CD222 and CD87 at similar density, only in ECs are the two proteins complexed in the membrane, and TGF-β is activated specifically by ECs but not by SMCs. Furthermore, we show that it is not whole Plg but its proteolytic fragment mini-plasminogen (mini-Plg) that is bound with high affinity by CD222, and that this binding is crucial for the activation of both TGF-β and apoptosis in ECs. Taken together, our data indicate a specific mechanism of TGF-β-activation-dependent apoptosis in ECs that is generated through physical association of CD87 and CD222.

Materials and Methods

Cells, antibodies and reagents

Both human umbilical-vein endothelial cells (HUVECs) and human umbilical-vein smooth-muscle cells (HUVSMCs) were isolated by collagenase digestion from human umbilical cords as described (Kroismayr et al., 2004). Cells were grown on gelatin-coated cell culture flasks at 37°C and 5% CO_2. HUVECs were cultured in M199, 20% serum, 50 mg ml^{-1} endothelial growth factor supplement (Technoclone, Vienna, Austria), antibiotics and 3 units ml^{-1} heparin (Roche). Primary cells were split in a 1:3 ratio and used up to the sixth passage. Mouse *CD222*$^{-/-}$ and wild-type (wt) fibroblasts were provided by E. Wagner (IMP, Vienna). *CD222*$^{-/-}$ fibroblasts stably expressing human CD222 or CD87 were prepared by retroviral infection (Leksa et al., 2002). 293 fibroblasts and THP-1 cells were from the ATCC. Mink lung epithelial cells (MLECs) stably transfected with a gene encoding TGF-β-responsive promoter (plasminogen-activator-inhibitor 1) fused to luciferase were from D. B. Rifkin (Department of Cell Biology, New York University Medical Center). HUVSMCs, THP-1 cells, MLECs and fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Monoclonal antibodies (mAbs) against CD222 (MEM-238), CD87 (H2, E4) and CD147 (MEM-M6-1), control mAbs AFP-01, mAb 2PG [against Plg kringle domains 1-3 (K1-3)] and mAb 4PG (against mini-Plg) were prepared by us. TGF-β and the polyclonal neutralizing antibodies against TGF-β isoforms [anti-TGF-β1 (AF-101-NA), anti-TGF-β2 (AB-112-NA) and anti-TGF-β3 (AB-244-NA)] were from R&D Systems (Minneapolis, MN). For confocal microscopy, we labeled the specific antibodies in our laboratories with Alexa Fluor 488 and 555. Peptides derived from the N-terminal part of CD222 (pepB, AVDTKNNVLYKINIAGSV; pepC HDLKTRT-YHSVVGDSVLRS,; pep12-30, TKNNVLYKINICGSVDIVQ) and control peptide (pepSCR, SVNCAIGSNGKVNYIKVNS) were produced by Genosphere, Paris, France. Plg and its fragments were from Technoclone.

Purification of proteins

CD222 was purified to homogeneity from lysates of human 293 fibroblasts or human THP-1 cells by affinity chromatography using CD222 mAb MEM-238 coupled to Sepharose. Plg, mini-Plg and K1-3 were either from Technoclone or isolated, with modifications, as described previously (Sottrup-Jensen, 1978). To obtain mini-Plg and K1-3, Plg was cleaved with porcine pancreatic elastase IV (Sigma, St Louis, MO, USA) coupled to CNBr-Sepharose beads and the cleavage products were fractionated on a heparin-Sepharose column (Sigma) using a 0-700 mM NaCl gradient.

In-vitro binding assay

10 μg ml^{-1} various molecules were coated in binding buffer (BB; 20 mM Tris-HCl, pH 7.5, 140 mM NaCl) on 96-well Falcon plates. The wells were blocked with 1% bovine serum albumin (BSA), washed and incubated for 1 hour with binding solution (BS; BB supplemented with 0.2% NP-40 and 2 μg ml^{-1} aprotinin) that contained 5 μg ml^{-1} CD222. Then, the wells were washed four times with BB and binding of CD222 was analysed by SDS-PAGE followed by immunoblotting using the anti-CD222 mAb MEM-238.

Surface-plasmon-resonance analysis

Kinetics and binding affinities were determined on a Bia 3000 optical biosensor (Biacore, AB, Sweden) and analysed using the BiaEvaluation Software. Two trays of the CM5 Biacore chip were directly loaded with purified CD222 by amine-coupling chemistry using *N*-hydroxysuccinimide/*N*-ethyl-*N*-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (Pierce, Rockford, IL, USA) at two concentrations, one with 4000 response units (RU) and the second with 1600 RU. As a negative control, a third tray was coupled with BSA (1000 RU) and a fourth was treated with coupling agents only. Purified proteins were dialysed against HEPES-buffered saline (HBS, pH 7.3) and ran over the chip in HBS containing 0.005% Tween-20 in different concentrations until equilibrium. Kinetic and equilibrium constants were determined from the CD222 binding curve after subtraction of the BSA binding curve using the BiaEvaluation Software. Kinetic constants were determined from the association and dissociation phases using a simple 1:1 Langmuir assumption. The association and dissociation constants were obtained from three different concentrations.

Immunoprecipitation

Cells were surface biotinylated using 0.5 mg ml^{-1} sulfo-NHS-LC-biotin (Pierce) in PBS. After incubation at 4°C for 1 hour, the reaction was stopped with 20 mM Tris-HCl, pH 8.2. Cells (2×10^7 ml^{-1}) were lysed for 30 minutes at 4°C in lysis buffer (20 mM Tris-HCl, pH 8.2, 140 mM NaCl) containing 1% NP-40 detergent (Pierce) and a set of protease inhibitors as described (Godar et al., 1999). Afterwards, the lysate was centrifuged for 5 minutes at 10,000 ***g***. The samples were then precleared on AFP-01/Sepharose (irrelevant IgG1 mAb AFP-01 coupled to CNBr-Sepharose) and subjected to immunoprecipitation using Sepharose beads coupled with specific mAb. Immunoprecipitates were washed four times with lysis buffer containing 1% NP-40 and analysed by immunoblotting. Membranes were blocked with 5% BSA and biotinylated proteins were visualized on the blot using a streptavidin-peroxidase conjugate (Amersham, Uppsala, Sweden) and chemiluminescence. CD222 was detected using MEM-238 and subsequent incubation with secondary antibodies coupled to peroxidase followed by chemiluminescence.

Flow cytometry and microscopy

Cells were detached, washed and incubated in PBS containing 1% BSA and the primary mAbs for 20 minutes on ice. After washing, cells were incubated with fluorescein isothiocyanate (FITC) conjugated sheep Fab'_2 anti-mouse IgG+IgM (H+L) antibodies (An der Grub, Kaumberg, Austria) for 20 minutes on ice, washed again and analysed by using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany). For microscopy, the cells were grown on gelatin-coated glass cover slips 1 day before staining. Subsequently, the cells were rinsed once and then fixed with 4% paraformaldehyde for 10 minutes. Following fixation, the cells were permeabilized with 0.1% Triton X-100 for 10 minutes and blocked using 4% beriglobin for 20 minutes. The cells were then incubated with fluorescently labeled antibodies for 20 minutes on ice and washed three times with

PBC containing 1% BSA. Imaging of the slides was performed using either a fluorescence microscope (Nikon HB-101101AF) or a confocal laser-scanning microscope (Zeiss LSM 510; Carl Zeiss). Isotype-matched antibodies were used as negative control.

Apoptosis assay

Cells were cultured in M199 medium containing 20% bovine calf serum, 2 ng ml^{-1} basic fibroblast growth factor (bFGF) and 100 mg ml^{-1} heparin. HUVECs were plated on a 96-well plate at 5×10^3 per well in gelatin-coated wells. After 24 hours, the culture medium was replaced by 150 μl of the same medium containing the assayed molecules and either 20% or 1% serum for high- or low-stringency conditions, respectively. After incubation for 24 hours, cell apoptosis was analysed using the Cell Death ELISA kit (Roche, Penzberg, Germany) detecting apoptotic histone-associated DNA fragments or by the annexin V-FITC kit (Calbiochem, La Jolla, CA). In some experiments, the cells were grown on a gelatin-coated cover slip and analysed by fluorescence microscopy. For fluorescence-activated cell sorting (FACS) analysis of annexin-V-stained cells, detached cells were collected and pooled with adhered cells harvested by a short trypsinization. The cells were then resuspended in the apoptosis-assay medium and incubated with FITC-conjugated annexin V for 20 minutes at room temperature in the dark. Propidium iodide (PI) was added shortly before FACS analysis to distinguish early-apoptotic cells from late-apoptotic or necrotic cells.

Cell-adhesion assay

Falcon 96-well plates were coated with 20 μg ml^{-1} various molecules in PBS. Afterward, the wells were washed with PBS and blocked with 1% BSA. Cells were trypsinized for exactly 1 minute at room temperature. Trypsinization was stopped by a tenfold volume of culture medium and the cells were washed two times with the same medium. After washing, cells were diluted in assay medium (RPMI medium without FCS) at a concentration of 10^5 cells ml^{-1}. 100 μl cell suspension was added per well and incubated for 40 minutes at 37°C. The wells were washed three times with assay medium. Cells attached to the wells were fixed with glutaraldehyde (0.25% v/v) for 10 minutes and stained for 30 minutes with a 0.1% solution of crystal violet in water. Unbound dye was washed off with water and cells were lysed overnight in a 0.5% Triton X-100. The absorbance of the released dye was measured at 595 nm. The number of adherent cells was determined from a standard curve.

TGF-β assay

Cells were cultured for 12 hours in assay medium: DMEM containing 2% bovine calf serum, 10 ng ml^{-1} bFGF and 100 μg ml^{-1} heparin (for HUVECs and HUVSMCs) or in RPMI with 2% FCS (for fibroblasts). To analyse total (active plus latent) TGF-β, the culture supernatant was heated for 10 minutes at 80°C to activate the LTGF-β. For analysis of the active TGF-β level, we used the untreated supernatant. The treated and untreated supernatants were diluted ten times in MLEC assay medium (DMEM, 0.1% BSA, 2 μg ml^{-1} aprotinin). The MLECs, containing a TGF-β-responsive Plg-activator-inhibitor 1 (PAI-1) promoter fused to the firefly luciferase gene (Nunes et al., 1995) were seeded in a 96-well plate (2×10^4 cells well^{-1}) 12 hours before the assay. The diluted supernatant from the assayed cells was added to the MLECs and incubated overnight. After incubation, the MLECs were washed, lysed and analysed for luciferase activity.

Statistical analysis

The experiments were performed at least three times in duplicate or triplicate, and the data were expressed as mean values with standard deviation. Statistical significance was evaluated using Student's *t*-test; a value of $P<0.05$ or $P<0.005$ (as indicated) was considered to be significant.

Results

TGF-β-mediated apoptosis in ECs

To test the effect of TGF-β as an inducer of apoptosis in HUVECs, we treated the cells with TGF-β isoforms 1, 2 and 3. In assay medium containing 20% serum, we observed the strongest response of TGF-β1 at a concentration of 10 ng ml^{-1}. The optimal concentration for TGF-β3 was also 10 ng ml^{-1}, whereas different concentrations of TGF-β2 did not yield a significantly different apoptotic response (although the highest response was seen at a concentration of 50 ng ml^{-1}). In the less-stringent medium with 1% serum, 2 ng ml^{-1} TGF-β1 strongly induced cell death (Fig. 1 and data not shown).

TGF-β activation by cells complexing CD87 and CD222

Because TGF-β is produced in the latent form, LTGF-β, it must be activated in order to induce biological responses. In our previous work, we have proposed a mechanism for LTGF-β activation by a protein complex made of purified proteins, whose core consists of CD87 and CD222. We found that, in addition to binding LTGF-β, CD222 also bound Plg, which (upon conversion to Plm by CD87-bound uPA) activated the adjacent LTGF-β (Godar et al., 1999). To discover whether this mechanism functions at the cellular level, we tested TGF-β activation in murine *CD222*$^{-/-}$ fibroblasts stably transduced with human CD87 and/or CD222 (Leksa et al., 2002). TGF-β activity was measured 12 hours after co-cultivation of cells with single-chain uPA (suPA) and/or Plg. Only cells expressing both CD87 and CD222 upregulated TGF-β activity in the presence of Plg. The presence of both Plg and suPA increased TGF-β activity twofold compared with Plg alone (Fig. 2). The increase of active TGF-β was dependent on the proteolytic activity of Plm, indicated by the sensitivity to the serine-protease inhibitor aprotinin.

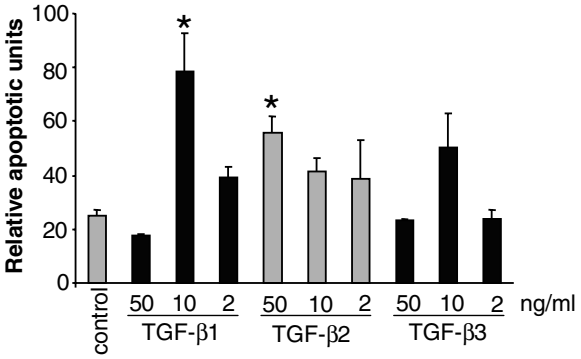


Fig. 1. TGF-β-induced apoptosis in ECs. HUVECs were cultivated in 96-well plates (5000 cells per well) in the presence of purified TGF-β (50 ng ml^{-1}, 10 ng ml^{-1} and 2 ng ml^{-1}) in assay medium. After incubation for 24 hours, the cells were washed with cultivation medium and lysed. Intact DNA was removed by centrifugation and the presence of soluble nucleosomes in the supernatant was detected by the Death ELISA kit (Roche). The relative apoptotic response was determined using a standard included in the kit.

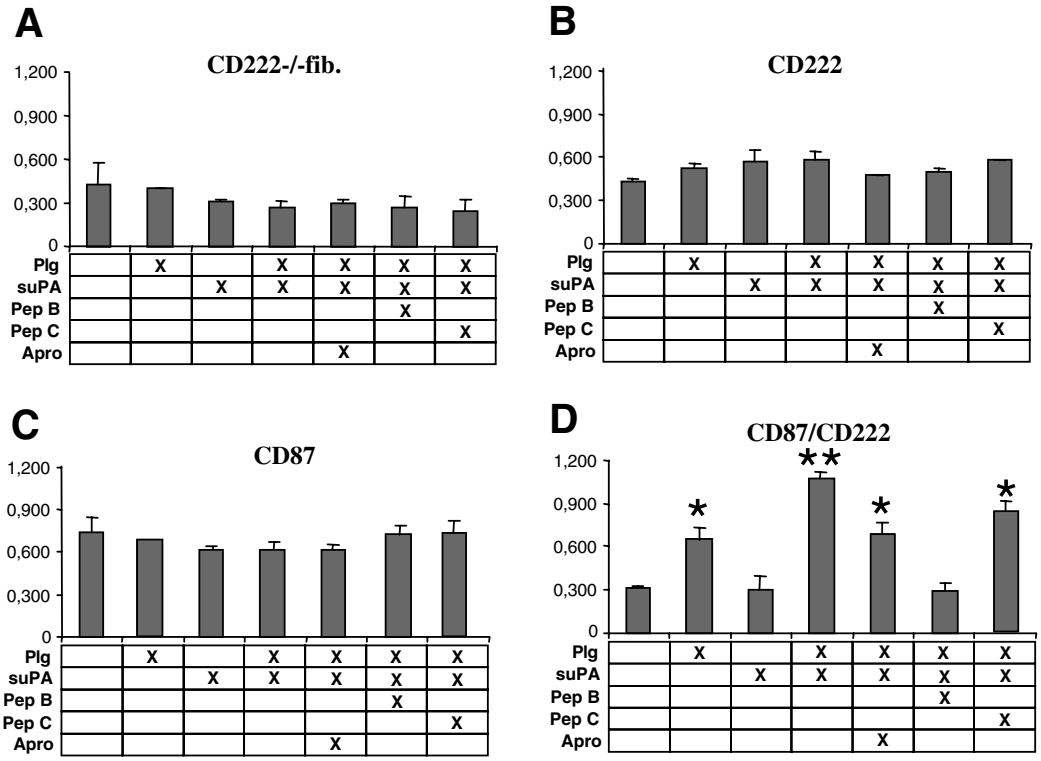


Fig. 2. Effect of CD87 and CD222 co-expression on TGF-β activation. Mouse *CD222*$^{-/-}$ fibroblasts (A) stably transduced with CD222 (B), CD87 (C) or both CD222 and CD87 (D) were cultivated in 96-well plates (5000 cells per well) and treated with 20 μg ml^{-1} Plg or 1 μg ml^{-1} suPA for 12 hours. The interfering agents were used in following concentrations: aprotinin (apro), 2 μg ml^{-1}; pepB or pepC, 20 μg ml^{-1}. After incubation, the culture supernatant was analysed for the presence of active TGF-β by the MLEC assay. Results are expressed as concentration of active TGF-β in ng ml^{-1}.

Previously, we have shown that a peptide derived from the N-terminal part of CD222 (pepB) inhibits co-precipitation of CD87 with CD222 (Leksa et al., 2002). To demonstrate the role of the CD87-CD222 association on LTGF-β activation, we compared the influence of pepB with that of a control peptide (pepC) that also is derived from the N-terminal part of CD222. The presence of pepB completely abolished TGF-β activity in supernatants from fibroblasts expressing both CD87 and CD222, whereas pepC had only a minor influence (Fig. 2). This indicates that the CD87-CD222 association is required for LTGF-β activation.

Mini-Plg is required for activation of TGF-β in ECs

To get a first indication whether the uPA-Plg-CD87-CD222 complex is also involved in TGF-β activation in ECs, we treated these cells with Plg and measured TGF-β activity in the cell culture supernatants. However, we did not find any sign of activation (Fig. 3A). By contrast, we did observe an inhibition of the total LTGF-β level after treatment with Plg, a phenomenon that we cannot explain at the moment.

Upon proteolytic cleavage by, for example, elastolytic metalloproteinases of tumor-associated macrophages (Cornelius et al., 1998) and neutrophil-secreted elastase (Scapini et al., 2002), Plg gives rise to several functionally potent fragments. Thus, we considered that one of these fragments might influence TGF-β activation in ECs. The family of fragments called angiostatin contains three to five kringle domains from Plg [kringles 1-3 (K1-3), kringles 1-4 (K1-4) or kringles 1-5 (K1-5)]. Angiostatin is secreted by certain experimentally induced tumors and is thought to inhibit angiogenesis in these tumors (Cao et al., 1998). The inhibitory effect of angiostatin on tumor angiogenesis seems to involve inhibition of endothelial cell-surface ATP synthase, integrin functions, pericellular proteolysis and cell migration, and/or induction of EC apoptosis (Moser et al., 1999; Scapini et al., 2002; Tarui et al., 2002; Tarui et al., 2001; Wajih and Sane, 2003). Another natural Plg fragment, consisting of the kringle 5 domain (K5) and the protease domain of Plg, is called mini-Plg.

To obtain K1-3, K4 and mini-Plg, we treated Plg with elastase and isolated the resulting proteolytic fragments as described (Sottrup-Jensen, 1978). As shown in Fig. 3A, we assessed the purity and activity of the isolated fragments by (1) Coomassie-stained SDS-PAGE, (2) western blotting and (3) measuring Plm activity with the colorimetric substrate S-2251 (Chromogenic, Milano) before and after activation with uPA. Only fractions of mini-Plg in which uPA increased Plm activity at least 50 times were used. When we tested the isolated Plg fragments for their TGF-β-activating capacity, we found that mini-Plg (but not angiostatin) was able to trigger TGF-β activation in ECs (Fig. 3B). As observed before with Plg, we also saw a reduction of the total LTGF-β level upon mini-Plg treatment. We also tested whether SMCs could release active TGF-β upon Plg or mini-Plg treatment but no activation was observed (Fig. 3C).

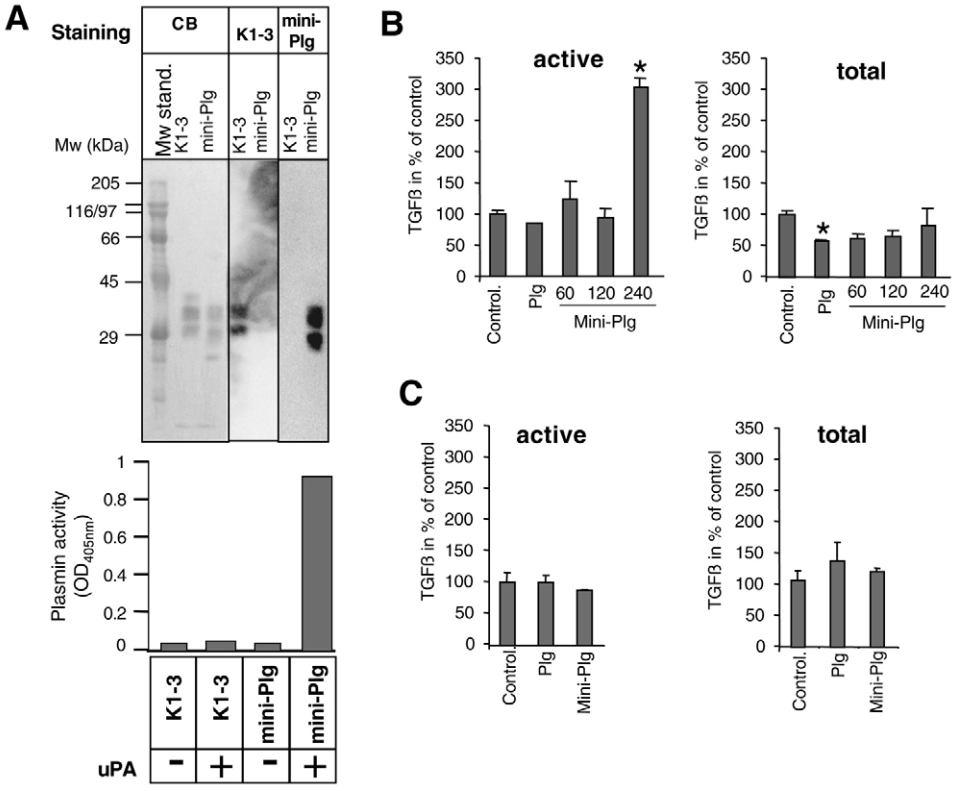


Fig. 3. Analysis of TGF-β activation in culture supernatants of ECs and SMCs by Plg fragments. (A) K1-3 and mini-Plg purity was analysed by Coomassie stain (CB) and by western blotting using the K1-3-specific mAb 2PG or the mini-Plg-specific mAb 4PG (top). The plasmin activity in the preparations was analysed using the plasmin-specific colorimetric substrate S-2251 before and after activation with active uPA (bottom). HUVECs (B) or HUVSMCs (C) were cultivated in 96-well plates (5000 cells per well) and treated with 240 nM Plg and 60 nM, 120 nM or 240 nM mini-Plg (B), or with 240 nM mini-Plg (C) in assay medium for 12 hours. For analysis of total TGF-β (LTGF-β plus active TGF-β), culture supernatants were heated for 10 minutes at 80°C. For analysis of active TGF-β alone, untreated supernatants were used. Active or total TGF-β was then analysed by the MLEC assay.

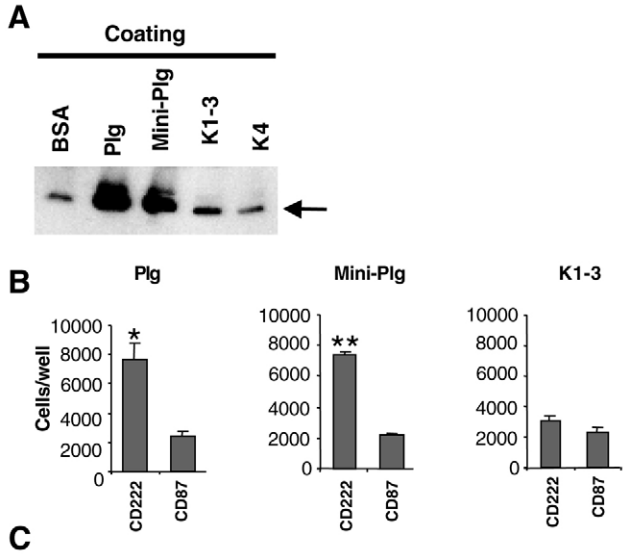
Mini-Plg is a ligand for CD222

We hypothesized, that if mini-Plg activated TGF-β by the CD222-dependent mechanism, it should also directly bind to CD222, analogously to Plg (Godar et al., 1999). To test this, we performed the following experiments. First, we did an in-vitro binding assay, which revealed that the only Plg fragment interacting with CD222 was mini-Plg (Fig. 4A). Second, we analysed the binding of mouse fibroblasts transduced with CD222 to Plg fragments immobilized on tissue-culture plates. Compared to *CD222*$^{-/-}$ fibroblasts expressing the CD87 molecule as control, CD222-expressing fibroblasts exhibited a significantly stronger binding to Plg and mini-Plg, but not to K1-3 (Fig. 4B). Finally, we analysed the binding of Plg and mini-Plg to CD222 by surface plasmon resonance (Fig. 4C). Plg-bound CD222 with an equilibrium constant of K_d=26.8×10^{-8} M. Mini-Plg exhibited a stronger (K_d=4.94×10^{-8} M) affinity to CD222. This can be attributed to the elevated association rate, because the dissociation rate was identical to that of Plg.

Mini-Plg induces apoptosis in ECs via TGF-β

Next, we studied whether the mini-Plg-activated TGF-β is involved in induction of apoptosis in ECs. We treated HUVECs with Plg fragments in an apoptosis assay. Mini-Plg induced apoptosis at a concentration of 240 nM (Fig. 5A). We did not observe apoptosis with K1-3, which is in contrast to previous studies (Dhanabal et al., 1999; Lucas et al., 1998; O'Reilly et al., 1994). To get more insight into how mini-Plg induced apoptosis in ECs, we treated the cells in the presence of the plasmin inhibitor aprotinin and the caspase-3 inhibitor DEVD-FMK. Both inhibitors downregulated mini-Plg-induced apoptosis, aprotinin partially and DEVD-FMK totally, showing that not only the proteolytic activity of mini-plasmin (mini-Plm) but also the classical caspase pathway was required for induction of apoptosis (Fig. 5A). To prove that the mini-Plg-induced apoptosis of ECs was mediated by TGF-β, we used a neutralizing anti-TGF-β antibody. As can be seen in Fig. 5A, this antibody reduced mini-Plg-induced apoptosis. Next, we double stained ECs with FITC-conjugated annexin V and PI. This method distinguishes between viable, early apoptotic and late apoptotic or necrotic cells. Fig. 5B shows representative cytograms for Plg- and mini-Plg-treated cells (24 hours). In the Plg-treated cells, we counted 15.6% early-apoptotic and 32.2% late-apoptotic or necrotic cells. The mini-Plg-treated cells displayed an increase in both early-apoptotic cells (19%) and late-apoptotic or necrotic cells (70.6%). Thus, mini-Plg induced cell death in ECs.

Finally, we tested whether the CD222-derived peptides could inhibit the mini-Plg-induced apoptosis. For that purpose, in addition to the already described peptides pepB and pepC (Leksa et al., 2002), we designed and prepared a new peptide from the N-terminal region of CD222, named pep12-30. As can be seen in Fig. 6, after a 24 hour treatment with mini-Plg, the cells showed morphological features such as membrane blebbing and shrinkage of the cell body, typical for apoptotic cells. Annexin-V staining was positive on those cells. When we co-treated the cells with the CD222-derived peptides pep12-30 and pepB, we observed a protective effect against mPlg-induced apoptosis. A scrambled peptide (pepSCR) and the CD222-derived control peptide pepC had no effect on EC apoptosis (Fig. 6A and not shown). It is important to realize



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Ligand	$k_a \times 10^4 M^{-1}s^{-1}$	$k_d \times 10^{-4}s^{-1}$	$K_d \times 10^{-8}M$
Plg	0.19 ± 0,00	5.07 ± 0,05	**26.80 ± 0,20**
Mini-Plg	0.96 ± 0,05	4.73 ± 0,26	**4.94 ± 0,58**

Fig. 4. Analysis of the mini-Plg/CD222 interaction. (A) Plg fragments were coated (10 μg ml^{-1}) on 96-well immunosorbent plates, which were blocked with 1% BSA. Then, the wells were incubated with purified CD222 for 4 hours at 4°C in BB. After washing twice with BB, bound CD222 was eluted using SDS-PAGE sample buffer and visualized by western blotting using the anti-CD222 mAb MEM-238. (B) Effect of CD222 expression on cell binding to mini-Plg. *CD222*$^{-/-}$ mouse fibroblasts expressing human CD222 or human CD87 were allowed to attach to 96-well plates coated with Plg, mini-Plg or K1-3. The number of cells displayed is the number of cells attached to wells coated with the indicated proteins after subtraction of the cell binding to BSA. (C) Determination of the binding affinity of Plg fragments to CD222 by surface plasmon resonance. Purified CD222 was immobilized on a Biacore chip. The equilibrium dissociation constant K_d was calculated from the ratio of dissociation rate constant k_d and association rate constant k_a. Standard deviation was obtained from three measurements.

that a lot of cells had detached upon the mini-Plg treatment and could have been found in medium. For the quantitative FACS evaluation, we pooled those cells with the adhered ones. Addition of pep12-30 but not the control peptide improved the cell viability by about 75% (Fig. 6B). When we performed a longer treatment with mini-Plg (48 hours), we found no early apoptotic cells but only dead cells. Again, pep12-30 reduced the number of the dead cells in a similar way to its effect in the shorter treatment (data not shown). These results further indicate the involvement of CD222 in the apoptosis induced by mini-Plg in ECs.

CD87 is complexed to CD222 in ECs

Our results show that, in contrast to SMCs, ECs are able to activate TGF-β via mini-Plg (Fig. 3B,C). If the CD87-CD222 complex mediated the mini-Plg-induced TGF-β activation in ECs then either the expression of CD87 or CD222, or their association should be altered in SMCs. Therefore, we assessed the surface expression of CD222 and CD87 on both cell types by immunofluorescence and flow cytometry. As shown in Fig. 7A, both cell types expressed CD222 and CD87 at comparable levels. Next, we performed a co-immunoprecipitation experiment: CD87 and CD222 were jointly isolated from lysates of ECs but not SMCs (Fig. 7B). Finally, we stained both ECs and SMCs with specific fluorescently labeled antibodies and analysed the staining by confocal microscopy. We observed most CD222 inside the cells; CD87 was present mainly on the surface. The receptors were found to be co-localized in ECs. By contrast, in SMCs, we found either no or much less co-localization than in ECs (Fig. 7C, white arrows), which is in line with the co-immunoprecipitation data. These results together with the data obtained using transductants that expressed CD87, CD222 or both (Fig. 2), indicate that the assembly of CD222 and CD87 is important for the activation of TGF-β.

Discussion

Apoptosis is involved in physiological angiogenesis, vasculogenesis and blood-vessel maintenance. TGF-β is one of the key modulators of vascular-cell apoptosis. In the present study, we propose a novel mechanism for TGF-β activation that is specific for ECs but not for SMCs. We provide evidence that the complex between CD222 and CD87 is important for the release of active TGF-β that, in turn, induces EC apoptosis in a caspase-dependent manner. The process is probably triggered by a specific binding of mini-Plg to CD222 and proteolytic activation of TGF-β by mini-Plm generated on the complex. These conclusions are supported by: (i) co-precipitation of CD222 with CD87 in ECs but not in SMCs; (ii) induction of TGF-β activation and apoptosis in ECs but not SMCs; (iii) production of active TGF-β by *CD222*$^{-/-}$ mouse fibroblasts only when co-expressing CD222 and CD87; (iv) inhibition of mini-Plg-induced apoptosis in ECs using CD222-derived peptides, a neutralizing anti-TGF-β antibody, the plasmin inhibitor aprotinin and a caspase-3 inhibitor; and (v) high-affinity binding of mini-Plg to CD222 as shown by adhesion of CD222 transductants to mini-Plg and an in-vitro-binding assay including surface-plasmon-resonance evaluation.

A question arising from our data is why mini-Plg, but not Plg, activates TGF-β and induces apoptosis in ECs. The concentration of Plg in human plasma is relatively high (2 μM) (Pollanen et al., 1991) and the affinity of Plg for CD222 is 26.80×10^{-8} M. This means that, under physiological conditions, the vast majority of surface-expressed CD222 might be occupied by Plg. Given that CD222 is complexed with CD87 on ECs and that ECs produce uPA (Booyse et al., 1988; Bykowska et al., 1982; Camoin et al., 1998; Takahashi et al., 1992; Tkachuk et al., 1996; van Hinsbergh et al., 1987) (for review, see Stepanova and Tkachuk, 2002) in an inactive form that is specifically activated in endothelial cells upon stimulation by vascular endothelial-cell growth factor (Prager et al., 2004), active TGF-β would always be formed upon an angiogenic stimulus and would thereby prematurely terminate angiogenesis. By contrast, mini-Plg is generated from Plg only under specific conditions. For instance, elastase secreted by neutrophils during perivascular inflammation can produce mini-Plg and thus could contribute to EC apoptosis (Boehme

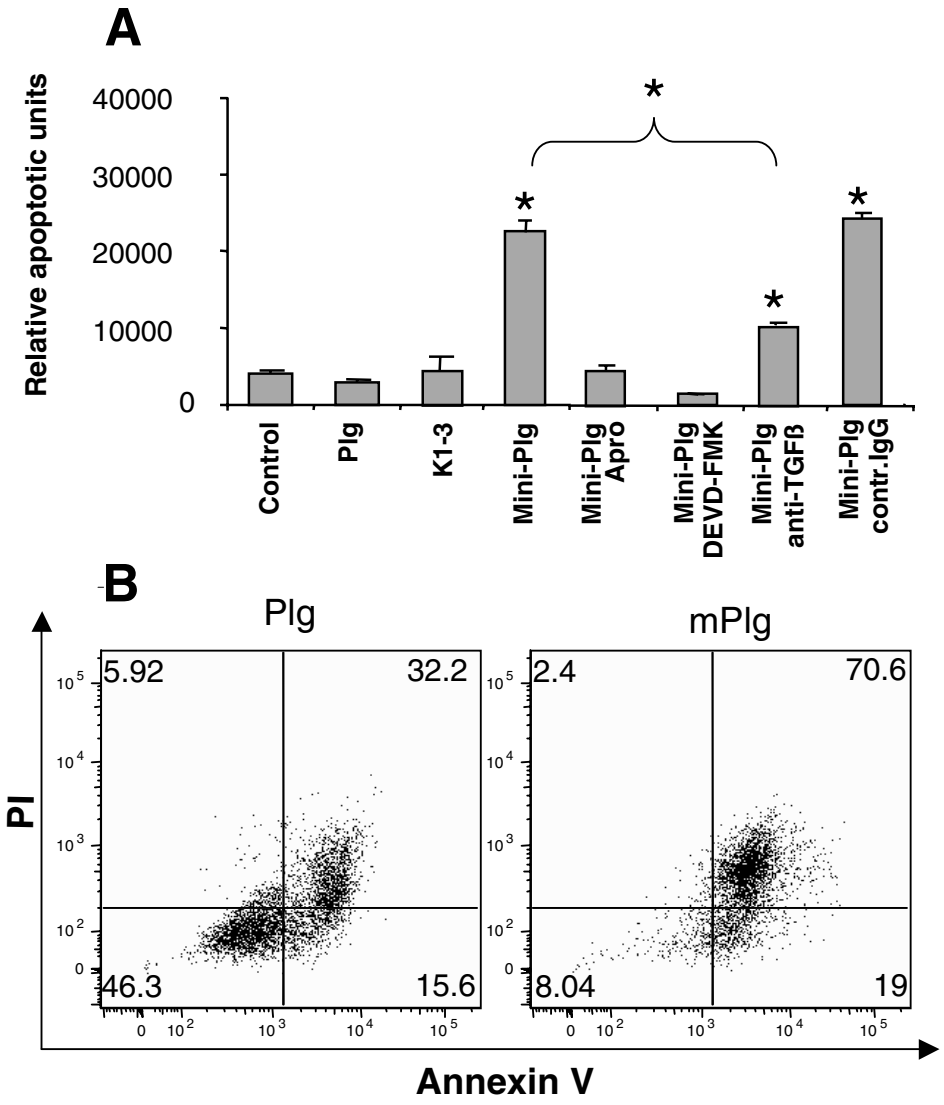

Fig. 5. Analysis of mini-Plg-induced apoptosis in ECs. (A) HUVECs cultivated in 20% serum medium in 96-well plates (5000 cells per well) were treated with 240 nM Plg or Plg fragments for 24 hours. After incubation for 24 hours, the cells were washed with cultivation medium and lysed. Intact DNA was removed by centrifugation and the presence of soluble nucleosomes in the supernatant was detected by the Death ELISA kit (Roche). The relative apoptotic response was determined using a standard included in the kit. HUVECs were treated with mini-Plg with or without the serine-protease inhibitor aprotinin (Apro, 10 μg ml^{-1}), the caspase-3 inhibitor DEVD-FMK (1 μM) or a neutralizing anti-TGF-β antibody (10 μg ml^{-1}) for 24 hours. (B) Cells were treated for 24 hours with 240 nM Plg or mini-Plg in medium containing 1% serum. After incubation, the detached cells were pooled with the trypsinized adherent cells and assayed for the occurrence of apoptosis by FACS analysis using an apoptosis detection kit (Calbiochem, La Jolla, CA, USA). Viable cells were annexin-V negative and PI negative; nonviable necrotic cells or late apoptotic were annexin-V positive and PI positive; early apoptotic cells were annexin-V positive and PI negative. Similar results were obtained in three different experiments.

et al., 2002; Machovich and Owen, 1989; Moroz, 1981; Yang et al., 1996). Mini-Plg can also be generated by cancer-cell-produced cathepsin D (Morikawa et al., 2000) or by a 24 kDa endopeptidase of bacterial origin (Lijnen et al., 2000). Furthermore, it can be generated by metalloelastase of tumor-associated macrophages and thus could contribute to apoptosis induction in ECs as an angiogenic inhibitor of tumors (Cornelius et al., 1998; Falcone et al., 1998).

The second product generated by this cleavage of Plg, angiostatin, is already known as an angiogenesis inhibitor silencing tumor angiogenesis (Soff, 2000). Because mini-Plg has a five-times-higher affinity for CD222 (4.95×10^{-8} M) than does Plg, mainly because of a higher association rate (Fig. 4), it has the potential to compete effectively with Plg and temporarily to replace it. However, there must be a further control mechanism for the activation of TGF-β on the surface of ECs by the CD87-CD222 complex, because fibroblasts overexpressing both CD87 and CD222 can activate TGF-β not only upon treatment with mini-Plg but also when treated with the parental protein Plg. It could be that fibroblasts but not ECs produce an additional factor controlling the conversion of Plg to mini-Plg. However, it is also possible that ECs possess some regulating mechanism on their surface that is not present on the surface of murine fibroblasts.

Another question is why fibroblasts expressing only CD87 produce a higher baseline of aprotinin-resistant active TGF-β than fibroblasts expressing both CD87 and CD222 (Fig. 2). A possible explanation is that CD87 also participates in a Plm-independent activation of LTGF-β. If this suggestion was right, CD222 would also serve as a regulator (indeed, an inhibitor) of this Plm-independent activation of LTGF-β.

In contrast to the literature, angiostatin K1-3 did not induce apoptosis in our cellular models. This controversy might be attributed to the difference in the experimental setup. (i) We used HUVECs, whereas published studies were done with more-sensitive bovine ECs (Dhanabal et al., 1999; O'Reilly et al., 1994). (ii) Our experiments were performed in 20% bovine calf serum and in the presence of bFGF, in contrast to the low-serum conditions used by others. (iii) Our experiments with K1-3 lasted for 24 hours, in comparison to several days for other experiments (Dhanabal et al., 1999; Lucas et al., 1998). All these differences made our pro-apoptotic assays more 'stringent'. However, despite the stringency of the conditions, mini-Plg was active under this setup and induced apoptosis.

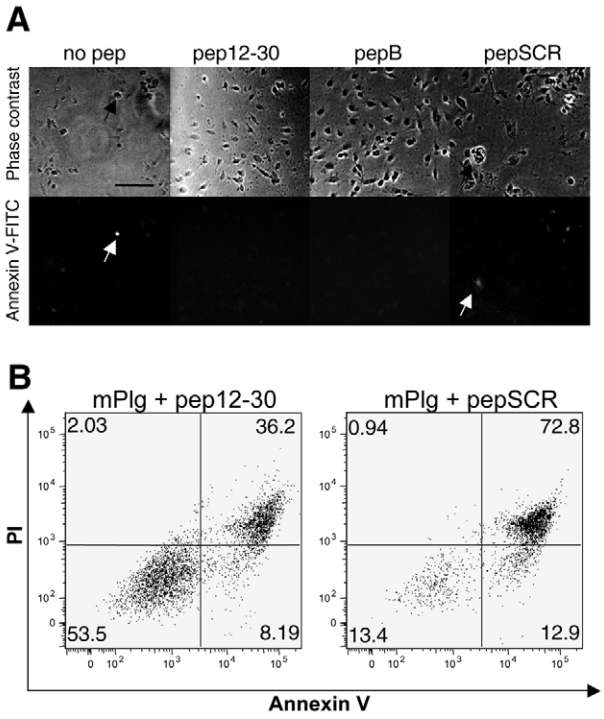

Fig. 6. Effects of CD222-derived peptides on mini-Plg-induced apoptosis in ECs. (A) HUVECs were incubated in medium containing 1% serum for 24 hours with mini-Plg (240 nM) in the presence of the CD222-derived peptides and control peptides (20 μg ml^{-1}), then stained with annexin-V/FITC and analysed under a fluorescence microscope. Apoptotic cells are indicated by arrows. Scale bar, 100 μm. (B) Cells were treated as in A. After incubation, the detached cells were pooled with the trypsinized adherent cells and assayed for the occurrence of apoptosis by FACS analysis using an apoptosis detection kit (Calbiochem, La Jolla, CA, USA). Viable cells were annexin-V negative and PI negative; nonviable necrotic cells or late apoptotic were annexin-V positive and PI positive; early apoptotic cells were annexin-V positive and PI negative. Similar results were obtained in three different experiments.

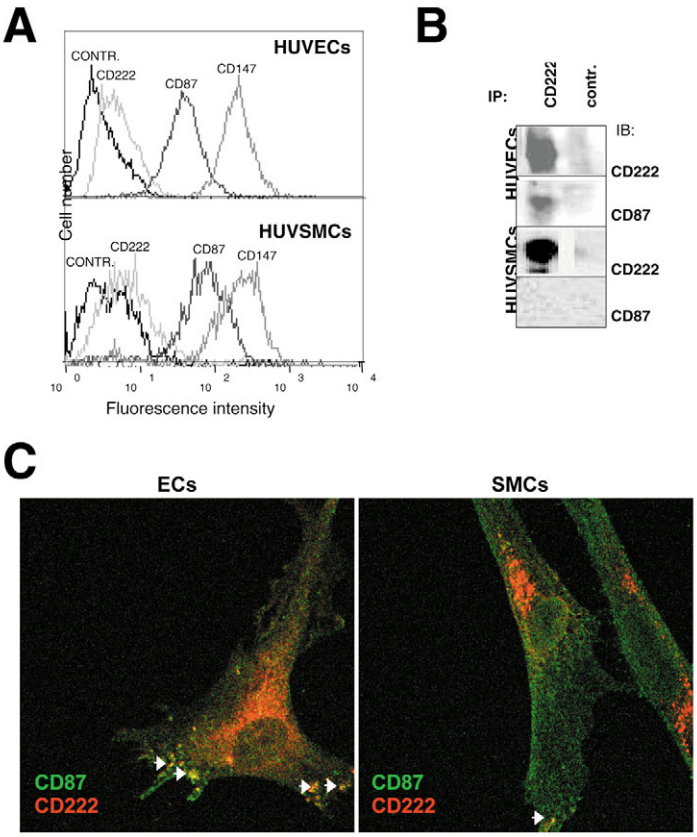

Fig. 7. Analysis of CD222 and CD87 expression and complex formation in ECs versus SMCs. (A) FACS analysis using specific mAbs. Cell-surface expression of CD222, CD87 and CD147 on HUVECs and HUVSMCs was analysed by immunofluorescence analysis and flow cytometry using the anti-CD222 mAb MEM-238, the anti-CD87 mAb C8 and the anti-CD147 mAb MEM-M6/1. (B) Surface-biotinylated HUVECs and HUVSMCs were subjected to immunoprecipitation using MEM-238. The precipitate was immunoblotted with MEM-238, the anti-CD87mAb H2 or the control mAb AFP-01. (C) HUVECs and SMCs were grown in gelatin-coated coverslips, washed, fixed, blocked and stained with the specific fluorescently labeled antibodies MEM-238/AF555 (CD222, red) and E4-AF488 (CD87, green). The slides were analysed under a confocal laser-scanning microscope. Colocalization structures (yellow) are indicated by arrows. All images show a region of 110×110 μm.

Mini-Plg consists of the protease domain and K5 of Plg. In addition to angiostatin (K1-3, K1-4, K1-5), K5 alone was also shown to be a potent inhibitor of endothelial cell growth (Cao et al., 1997). A recent report provides evidence for a specific receptor responsible for the K5 effect (Gonzalez-Gronow et al., 2003). However, the key part of mini-Plg for activation of TGF-β and apoptosis induction seems to rely on the protease domain and the proteolytic activity rather than on the K5 binding. The observed effects were sensitive to aprotinin, a serine-protease inhibitor that blocks Plm activity. CD222 is already known as a proapoptotic mediator by sorting granzyme B to the site of caspase 3, which (upon proteolytic activation by granzyme B) induces the apoptotic machinery. Based on these data and our finding that mini-Plg-induced apoptosis is blocked by a caspase-3 inhibitor, one could assume that CD222 is also targeting mini-Plg to caspase 3, which is activated then by mini-Plm (Motyka et al., 2000). However, the mini-Plg-induced apoptosis appears to act via proteolytic release of active TGF-β from LTGF-β, as indicated by partial neutralization of apoptosis via an anti-TGF-β antibody (Fig. 5A).

Collectively, we conclude that uPA bound to CD87 activates CD222-bound mini-Plg to the active protease mini-Plm. The close proximity of mini-Plm to the CD222-bound LTGF-β enables release of the active cytokine TGF-β. This mechanism provides a temporarily and spatially confined activation of this potent cytokine. The process seems to be further controlled by the cleavage of Plg to mini-Plg, for example with elastolytic metalloproteinases of tumor-associated macrophages (Cornelius et al., 1998), neutrophil-secreted elastase (Scapini et al., 2002) or cancer-cell-produced cathepsin D (Morikawa et al., 2000) (Fig. 8). Furthermore, although they express similar amounts of CD87 and CD222 on their surface, HUVECs can activate TGF-β in response to mini-Plg but cannot HUVSMCs. CD87 and CD222 are complexed on the surface of HUVECs but not on the surface of HUVSMCs, indicating a further level

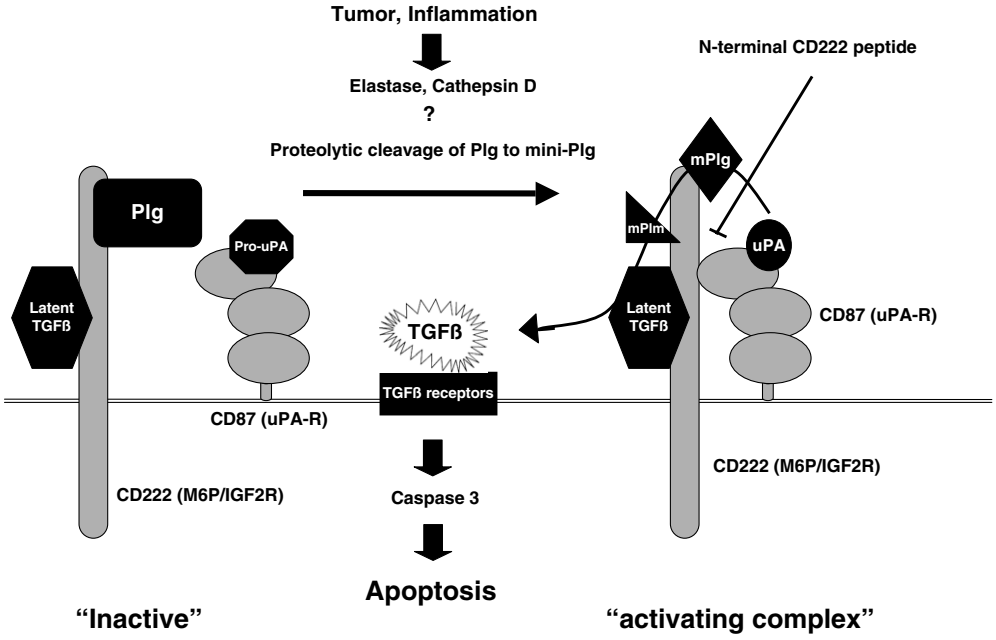

Fig. 8. Model of min-Plg-mediated apoptosis in ECs. Our findings indicate that the complex between CD87 and CD222 is a platform for the activation of LTGF-β. We have shown that mini-Plg, a proteolytic fragment of Plg, can induce TGF-β activation followed by apoptosis in ECs. Based on our data and the studies of other laboratories (Cornelius et al., 1998; Godar et al., 1999; Morikawa et al., 2000; Scapini et al., 2002), we propose that, under certain pathological conditions, such as inflammation or cancer, the inactive proenzyme Plg is converted to mini-Plg upon binding to CD222 (e.g. by elastase or cathepsin D). Because CD222 associates with CD87 in ECs, the CD87-bound uPA can convert the CD222-bound mini-Plg to the active protease mini-Plm. This leads to partial digestion of LTGF-β bound to CD222, followed by the release of the active TGF-β, inducing apoptosis in ECs.

of control of this important cytokine. The importance of the CD222-CD87 interaction in this mechanism is strengthened by the observation that peptides derived from the N-terminus of CD222 that disrupt the interaction of CD87 with CD222 (Leksa et al., 2002) also inhibit both TGF-β activation and mini-Plg-induced apoptosis (Figs 2, 6).

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