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Novel Fibrogenic Pathways Are Activated in Response to Endothelial Apoptosis: Implications in the Pathophysiology of Systemic Sclerosis

Patrick Laplante,* Marc-André Raymond,* Gabrielle Gagnon,* Normand Vigneault,* A. Marie-Josée Sasseville,* Yves Langelier,* Monique Bernard,† Yves Raymond,† and Marie-Josée Hébert2*

Apoptosis of endothelial cells (EC) is appreciated as a primary pathogenic event in systemic sclerosis. Yet, how apoptosis of EC leads to fibrosis remains to be determined. We report that apoptosis of EC triggers the release of novel fibrogenic mediators. Medium conditioned by apoptotic EC (SSC) was found to inhibit apoptosis of fibroblasts, whereas medium conditioned by EC in which apoptosis was blocked (with either pan-caspase inhibition or Bcl-xL overexpression) did not. PI3K was activated in fibroblasts exposed to SSC. This was associated with downstream repression of Bim-EL and long-term up-regulation of Bcl-xL protein levels. RNA interference for Bim-EL in fibroblasts blocked apoptosis. SSC also induced PI3K-dependent myofibroblast differentiation with expression of α-smooth muscle actin, formation of stress fibers, and production of collagen I. A C-terminal fragment of the domain V of perlecan was identified as one of the fibrogenic mediators present in SSC. A synthetic peptide containing an EGF motif present on the perlecan fragment and chondroitin 4-sulfate, a glycosaminoglycan anchored on the domain V of perlecan, induced PI3K-dependent resistance to apoptosis in fibroblasts and myofibroblast differentiation. Human fibroblasts derived from sclerodermic skin lesions were more sensitive to the antiapoptotic activities of the synthetic peptide and chondroitin 4-sulfate than fibroblasts derived from normal controls. Hence, we propose that a chronic increase in endothelial apoptosis and/or increased sensitivity of fibroblasts to mediators produced by apoptotic EC could form the basis of a fibrotic response characterized by sustained induction of an antiapoptotic phenotype in fibroblasts and persistent myofibroblast differentiation.


Systemic sclerosis is an autoimmune disorder characterized by early endothelial cell (EC) damage followed by development of cutaneous as well as visceral fibrosis (1). Fibrosis occurs in association with acquisition of an antiapoptotic phenotype in fibroblasts and differentiation of fibroblasts toward the myofibroblastic phenotype (2, 3). The myofibroblast, a specialized and contractile type of fibroblast, is a normal cellular constituent of healing tissues. It is characterized by the presence of stress fibers that contain α-smooth muscle actin (αSMA) and increased production of collagen I and III (2). During normal wound healing, myofibroblasts play a central role in extracellular matrix (ECM) deposition and subsequent wound contraction. After completion of tissue or vascular remodeling, repair is terminated by clearance of myofibroblasts and superfluous fibroblasts through apoptosis (2–4). In fibrotic disorders, which in many ways resemble a sustained form of repair, healing fails to terminate due to lack of apoptosis in fibroblasts and myofibroblasts (1, 2, 4).

Mounting evidence suggests that apoptosis of EC is a primary pathogenic event in systemic sclerosis (1, 5). Increased endothelial apoptosis was shown to represent the earliest microvascular abnormality in an animal model of systemic sclerosis and to precede fibrotic changes (5). Anti-EC Abs, found in a majority of sclerodermitic patients, are known to induce EC apoptosis both in vitro (6) and in vivo (7). Studies in early inflammatory human sclerodermic lesions have also supported the contention that endothelial apoptosis is an initial triggering phenomenon in systemic sclerosis (5). Yet, the mechanistic interplay between EC apoptosis and fibrogenesis remains largely undefined.

We showed previously that endothelial apoptosis triggers the release of novel paracrine mediators, which include a C-terminal fragment of perlecan (8). These mediators, in turn, increase proliferation and inhibit apoptosis of EC and vascular smooth muscle cells (8, 9). Based on these results, we proposed that paracrine repair pathways are “built in” the apoptotic program to prevent widespread apoptotic cell death and initiate repair after cell deletion by apoptosis (8). In the present work, we evaluated whether mediators released by apoptotic EC favor development of an antiapoptotic phenotype in fibroblasts and myofibroblast differentiation. We also evaluated whether these mediators activate the PI3K/Akt pathway in fibroblasts, because this signaling pathway has been recently implicated in resistance to apoptosis and myofibroblast differentiation of human systemic sclerosis skin fibroblasts (1). Finally, we evaluated whether fibroblasts derived from
Materials and Methods

**Patients and cell lines**

HUVECs were obtained from Clonetics, grown in EC basal medium (Clonetics) and used at passages 2–4. WI-38 human fibroblasts from normal embryonic lung tissue were obtained from American Type Culture Collection, grown in fibroblast basal medium (Cambrex) supplemented with 10% inactivated FBS (Medicorp) and used at passages 2–17. Primary human fibroblasts were isolated from adult skin biopsies obtained from areas of clinically lesional skin of patients with systemic sclerosis or normal adult skin as previously described (10). This procedure was approved by the institutional review board. Fibroblasts were cultured in DMEM (Wisent) supplemented with 10% inactivated FBS and used at passages 3–8.

**Screening for apoptosis with fluorescence microscopy**

Fluorescence microscopy of unfixed/unpermeabilized adherent cells stained with Hoechst 33342 (2'-4'-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2'-1H-benzimidazole (HT) and propidium iodide (PI) was used as described in our previous work (8, 9, 11–13). In brief, cells were grown to confluence in 24-well polycarbonate culture plates (BD Biosciences). HT (1 μg/ml) was added for 10 min at 37°C and cells were washed with PBS. PI was added to a final concentration of 5 μg/ml immediately before fluorescence microscopy analysis (excitation filter, λ = 360–425 nm). The percentages of normal, apoptotic, and necrotic cells adherent to the dish were estimated by an investigator blinded to the experimental conditions. Viable cells display normal nuclear and cytoplasmic morphology and stain blue. Early apoptotic cells are characterized by cell shrinkage, nuclear condensation, and preservation of plasma membrane integrity. Chromatin condensation is associated with enhanced fluorescence for HT (bright blue), whereas preservation of cell membrane integrity precludes PI staining. Late apoptotic cells (also called secondary necrosis) are characterized by the presence of characteristic apoptotic nuclear changes and loss of cell membrane integrity associated with PI staining. Primary necrotic cells are characterized by increased cell size, absence of chromatin condensation, and disruption of cell membrane integrity.

**Immunoblotting**

Proteins were extracted, separated by electrophoresis, transferred to nitrocellulose membranes, and probed as we described previously (8, 9, 13). The following Abs were used: anti-Bcl-xL (BD Pharmingen), anti-p53 (Santa Cruz Biotechnology), anti-Bim-EL (Calbiochem), anti-Bcl-2 (BD Pharmingen), anti-αSMA (Sigma-Aldrich), anti-desmin (Sigma-Aldrich), anti-cIAP1/cIAP2/XIAP (R&D Systems), anti-phospho-Akt (Ser⁴⁷³), and anti-Akt (Cell Signaling Technology). All membranes were stripped (2% SDS, 100 mM 2-ME, and 62.5 mM Tris-HCl (pH 6.7)) at 50°C for 30 min with gentle shaking and reprobed with anti-α-tubulin mAb (Oncogene) as a control for protein loading.

**Immunofluorescence for characterization of myofibroblast differentiation**

**Immunofluorescence: αSMA.** Cells grown on glass coverslips were rinsed with PBS, fixed in methanol (5 min) and acetone (10 min), and dried at room temperature followed by three washes with PBS before permeabilization and after each subsequent step. Permeabilization was performed in PBS/Tween 80 (1%) for 15 min. Coverslips were then blocked with PBS/BSA (3%) for 15 min and incubated with mouse monoclonal anti-αSMA Ab (Sigma-Aldrich) and FITC-labeled anti-mouse Ab (Jackson ImmunoResearch Laboratories). Cells were visualized at room temperature using a Nikon Eclipse-E600 microscope and MetaMorph-4.6.9 acquisition software.

**Immunofluorescence: stress fibers.** Cells were grown on glass coverslips, rinsed with PBS, and fixed with 2% formaldehyde/4% sucrose in PBS. Cells were washed three times in PBS before permeabilization and after each subsequent step. Permeabilization was performed with 50 nM NH₄Cl and 0.3% Triton X-100 in PBS for 15 min. Coverslips were blocked with PBS/BSA (3%) for 30 min at room temperature. Cells were then visualized at room temperature using a Nikon Eclipse-E600 microscope and MetaMorph-4.6.9 acquisition software.

**Adenovirus infection**

The adenovirus recombinants AdTREx-FLAG-Bcl-xL and AdTREx-GFP expressing, respectively, the antiapoptotic mouse Bcl-xL and GFP were kindly provided by G. Shore (McGill University, Montreal, Quebec, Canada). HUVEC were grown to confluence prior to the experiment. Cells were either mock-infected or infected in normal medium using conditions to maximize viral adsorption at a multiplicity of infection of 20 PFU/cell for 18 h as previously described (14). After the adsorption period, the viral inoculum was replaced by fresh normal medium, and cells were grown for 48 h before exposition to either normal or serum-free medium for 4 h.

**RNA interference**

WI-38 fibroblasts were plated onto 6-well plates and 24-well plates at 100,000 and 20,000 cells per well, respectively. After 20 h, cells were transfected with double-stranded RNA-DNA hybrids at a final concentration of 200 nM annealed oligo using Oligofectamine (Invitrogen Life Technologies). After 45 h of transfection, cells were placed in experimental conditions for 24 h, followed by evaluation of apoptosis with fluorescence microscopy or evaluation of Bim-EL protein levels by Western blotting. Oligonucleotides were obtained from Dharnacon Research. Bim-EL sense was 5'-GACCAGAAGGUGAUGAAUUGUdT(T)-3'. Bim-EL antisense was 5'-CAAUUGCUACCCUUCCGGUCd(TT)-3'. Bim-EL antisense was 5'-GGCGCUAAUCAUGGACCUd(TT)-3', and control antisense was 5'-AAUACAGCAUGUACACGd(TT)-3' as previously reported (15).

**ELISA for estimation of secreted collagen type I levels and active TGF-β1 levels**

**Collagen type I.** A competitive ELISA was used to estimate the levels of collagen type I present in cell culture medium before and after exposure to WI-38 fibroblasts for 7 days. One hundred microliters of these cell culture media were preincubated overnight at 37°C on a 96-well plate with 1 µg/ml anti-type I collagen Ab (Biodies International) diluted in PBS/BSA (1%). At the same time, a second 96-well polystyrene plate was coated with 100 µl of purified collagen type I (5 µg/ml; Biodies International) diluted in 20 mM Na₂CO₃ (pH 9.6) for 2 h at room temperature and then overnight at 4°C. The coated plate was washed three times with PBS/Tween 20 (0.05%) and blocked with 200 µl of PBS/BSA (1%) for 2 h. The preincubated samples were added to the coated plate for 1 h at room temperature. The Abs that did not form complexes with the secreted collagen present in culture medium will bind to type I collagen coated on the second 96-well plate. Controls with blocking solution (PBS/BSA (1%) alone and anti-type I collagen Ab (1 µg/ml) alone were also included. Then, the plate was washed three times following addition of the peroxidase-conjugated secondary Ab (anti-rabbit; BioSource International) at a dilution of 1/20,000 in PBS/BSA (1%) for 90 min at room temperature. The peroxidase substrate (0.003% H₂O₂; 0.4 mg/ml α-phenylenediamine in 0.1 M citrate buffer (pH 6.0) was added after three washes, and the plate was kept in the dark for 30 min. Collagen concentration in the competitive assay will be inversely proportional to the level of unbound anti-collagen I Abs and consequently to the intensity of the peroxidase reaction. The reaction was stopped by adding 2 M H₂SO₄ (50 µl/mill), and the absorbance was measured at 490 nm. The concentration of collagen type I was estimated using reference samples of purified collagen type I.

**TGF-β1.** Levels of active TGF-β1 were measured using a commercially available active-TGF-β1 Immunonoassay kit (BioSource International) according to the protocol provided by the manufacturer.

**Reagents**

Peptide no. 1 homologous to the cholesterol core of the C-terminal fragment of perlec in medium conditioned by apoptotic EC and containing the EGF motif (aa 4147–4182: CQLREPCHLGTCGTQRCCLCPFGS PRCQ CGSHGH) and the control peptide (peptide no. 2) homologous to the C-terminal end of perlec (aa 4351–4391: GTGCVKNLVLHSARP GAFFPQPPLIDLQHRAGANTRPCPS) were synthesized at the Biotechnology Research Institute of Canada (Montreal, Quebec, Canada). The methodology used for synthesis of these peptides is based on an Fmoc-based solid-phase strategy. Purification was made using a Vydac 218TP C18 reverse-phase HPLC column (Grace-Vydac) where a sample of each of the synthetic peptides was diluted in 20% acetonitrile/H₂O. These mixtures were injected in the column before elution with 20–60% of solution B during 120 min at a flow rate of 13 ml/min (solution A: 0.1% trifluoroacetic acid/H₂O; solution B: 0.1% trifluoroacetic acid/acetonitrile). The eluates corresponding to the main peaks were collected, and the solvent was removed by lyophilization. The amino acid analysis was obtained using a PerkinElmer API III SCIEX mass spectrometer to obtain synthetic...
peptides pure at 100%. LY294002 and chondroitin 4-sulfate (CS) were purchased from Calbiochem. zVAD-FMK was purchased from R&D Systems. All other reagents were from Sigma-Aldrich.

Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed using Student’s t test or ANOVA, as appropriate. A value of \( p < 0.05 \) was considered significant for all tests.

Results

Apoptosis of EC triggers the release of antiapoptotic factors active on fibroblasts

Apoptosis of HUVEC was induced with serum starvation for 4 h to generate serum-free medium conditioned by apoptotic EC (SSC) as we described previously (8, 9, 12). In turn, this conditioned medium was used to serum starve human embryonic pulmonary fibroblasts (WI-38) for 24 h. Apoptosis was significantly
reduced in fibroblasts exposed to SSC compared with fresh serum-free medium (SS) (Fig. 1A). Inhibition of apoptosis in fibroblasts was also documented after long-term (7-day) exposure to SSC (Fig. 1A). To test whether mediators produced by serum-starved EC induce a broad antiapoptotic phenotype, fibroblasts were exposed to bleomycin in presence of SSC or SS for 24 h. Bleomycin is a DNA-damaging agent associated with development of systemic sclerosis in animals and humans (16). Fibroblasts exposed to bleomycin in presence of SSC showed a significantly decreased apoptotic response as compared with fibroblasts exposed to bleomycin in serum-free medium (Fig. 1B).

To test whether biologically active mediators present in SSC are released by apoptotic EC, apoptosis of EC during conditioning was blocked with a pancaspase inhibitor. HUVEC were exposed to zVAD-FMK (100 μM) or vehicle (DMSO) for 2 h. zVAD-FMK and vehicle were then removed, and HUVEC were washed and serum starved for 4 h. We showed previously that preincubation with zVAD-FMK significantly decreased the percentage of apoptotic EC after 4 h of serum starvation, whereas preincubation with DMSO did not (8). Serum-free medium conditioned by EC in which caspase activation was blocked (SSC-zVAD) failed to induce resistance to apoptosis in fibroblasts (Fig. 1C). These results suggest that antiapoptotic mediators active on fibroblasts are released by apoptotic EC downstream of caspase activation. To further support this conclusion, HUVEC were infected with an adenovirus recombinant expressing Bcl-xL or with a control adenovirus recombinant expressing GFP. Mock-infected HUVEC were also included as an additional control. Forty-eight hours after infection, HUVEC were exposed to normal or serum-free medium for 4 h, followed by evaluation of apoptosis. Bcl-xL overexpression blocked development of apoptosis in serum-starved HUVEC (Fig. 1D). Serum-free medium conditioned by HUVEC overexpressing Bcl-xL did not inhibit apoptosis of fibroblasts, whereas serum-free medium conditioned by HUVEC infected with the control adenovirus recombinant significantly inhibited development of apoptosis in fibroblasts (Fig. 1E). These results demonstrate that apoptosis of EC is necessary for the release of antiapoptotic factors active on fibroblasts.

**Central role for PI3K in development of an antiapoptotic phenotype in fibroblasts**

Fibroblasts exposed to SSC for 1 and 4 h showed increased phosphorylation of Akt compared with normal medium (N) and SS (Fig. 2A). Coincubation of fibroblasts for 24 h with SSC and LY294002, a biochemical inhibitor of PI3K, blocked the development of resistance to apoptosis (Fig. 2B). The dose-dependent activity of LY294002 was evaluated in our system. We used the lowest concentration (5 μM) associated with inhibition of the antiapoptotic activity of SSC (data not shown). This concentration falls within the lower range of concentrations reported to inhibit PI3K in fibroblasts (17, 18). We went on to characterize the molecular mechanisms of resistance to apoptosis activated downstream of PI3K in fibroblasts exposed to mediators released by apoptotic EC. Protein levels of p53, cIAP1, cIAP2, xIAP, and Bcl-2 were unaltered in fibroblasts exposed to SSC for up to 7 days as compared with SS (data not shown). Protein levels of the proapoptotic molecule Bim-EL increased in fibroblasts exposed to SS for 4 h (data not shown) and 24 h (Fig. 2C), whereas SSC prevented the up-regulation of Bim-EL (C). Coincubation of fibroblasts with LY294002 in presence of SSC restored the up-regulation of Bim-EL (Fig. 2C) and the apoptotic response (B). Blocking Bim-EL expression with RNA interference in fibroblasts exposed to SS inhibited development of apoptosis (Fig. 2, D and E), demonstrating the mechanistic importance of Bim-EL overexpression in fibroblast apoptosis. RNA interference for Bim-EL in fibroblasts exposed to SSC did not reduce further Bim EL expression or apoptosis (Fig. 2, D and E), suggesting that Bim-EL activity was maximally reduced in presence of SSC. These results suggest that mediators released by apoptotic EC inhibit apoptosis of fibroblasts through PI3K-dependent Bim-EL repression.

On the long term (4 and 7 days), resistance to apoptosis mediated by SSC was associated with a progressive increase in the protein levels of the antiapoptotic molecule Bcl-xL (Fig. 2F). Coincubation of fibroblasts with SSC and LY294002 for 7 days blocked the development of a long-term antiapoptotic phenotype in fibroblasts (Fig. 2G). LY294002 was also found to attenuate Bcl-xL up-regulation in fibroblasts exposed to SSC for 7 days (Fig. 2H). Hence, these results suggest that mediators released by apoptotic EC induce resistance to apoptosis in fibroblasts through PI3K-dependent sequential modulation of Bim-EL and Bcl-xL protein levels.

**A fragment of the domain V of perlecan induces resistance to apoptosis in fibroblasts**

We showed previously that a C-terminal fragment of the domain V of perlecan, which comprises one EGF motif and an anchoring site for CS, is one of the bioactive mediator(s) present in SSC (8). In the present work, we found that apoptosis was inhibited in fibroblasts exposed for 24 h to SS supplemented with either CS or a synthetic peptide containing the EGF motif present of the perlecan fragment (peptide no. 1), whereas fibroblasts exposed to a control peptide (peptide no. 2) failed to show resistance to apoptosis (Fig. 3A). LY294002 blocked the antiapoptotic response induced by CS and peptide no. 1 (Fig. 3B). Bim-EL up-regulation was significantly decreased in fibroblasts exposed for 24 h to SS supplemented with either CS or peptide no. 1 (Fig. 3C).

On the long term, fibroblasts exposed for 7 days to SS supplemented with CS developed an antiapoptotic phenotype, whereas fibroblasts exposed to peptide no. 1 for 7 days failed to show resistance to apoptosis (Fig. 3D). To prevent degradation and potential loss of activity of the synthetic peptides, culture medium exposed to fibroblasts were changed every 48 h. This time point was chosen as we found that the antiapoptotic activity of the synthetic peptide no. 1 (0.1 μg/ml) on fibroblasts was maintained over 48 h (percentage of apoptotic fibroblasts after 48 h: SS, 3.38 ± 0.38%; SS plus synthetic peptide no. 1, 1.54 ± 0.32%; p = 0.001; n = 17). Resistance to apoptosis was blocked in fibroblasts exposed for 7 days to CS in presence of LY294002 (Fig. 3E), suggesting that the antiapoptotic phenotype induced by CS also develops downstream of PI3K activation. Fibroblasts exposed to serum-free medium supplemented with CS for 7 days showed increased Bcl-xL protein levels (Fig. 3F), whereas exposure to peptide no. 1, which did not inhibit apoptosis of fibroblasts, failed to up-regulate Bcl-xL protein levels (F). These results suggest that motifs present on the C-terminal fragment of perlecan can recapitulate the molecular pattern of apoptosis inhibition induced by SSC.

**Mediators produced by apoptotic EC induce myofibroblast differentiation**

We evaluated whether mediators produced by apoptotic EC also regulate myofibroblast differentiation. Fibroblasts exposed to SSC for 7 days showed increased protein levels of αSMA and desmin compared with fibroblasts maintained in N and SS (Fig. 4A). Production of collagen type I was increased by 2-fold in fibroblasts exposed to SSC compared with fibroblasts exposed to SS for the
same duration (Fig. 4B). Increased immunostaining for αSMA and increased formation of stress fibers were found in fibroblasts exposed for 7 days to SSC compared with N and SS (Fig. 4, C and D). SS supplemented with TGF-β1, a classical inducer of myofibroblast differentiation (19, 20), is shown as a positive control (Fig. 4, C and D). Inhibition of PI3K with LY294002 (5 μM) or vehicle (DMSO), *p < 0.05 vs N; & p < 0.008 vs SSC and SSC-DMSO, representative of five independent experiments. C, Immunoblotting analysis. Increased protein levels of Bim-EL in WI-38 fibroblasts exposed to SSC in presence of LY294002 (5 μM) for 24 h, compared with vehicle (DMSO) and SSC alone. These results are representative of four independent experiments. Two images from the same gel (with corresponding α-tubulin) are shown. D, Percentage of apoptotic cells in WI-38 fibroblasts transfected with Bim-EL small interfering RNA (siRNA) or control before exposure to N, SS, and SSC for 24 h; *, p < 0.02 vs N; & p < 0.03 vs SS + Oligofectamine and SS + control siRNA, representative of six experiments. E, Immunoblotting analysis. Decreased protein levels of Bim-EL in WI-38 fibroblasts exposed to SS + Bim-EL siRNA compared with SS + Oligofectamine and SS + control siRNA. These results are representative of five independent experiments. F, Immunoblotting analysis. Increased protein levels of the antiapoptotic molecule Bcl-xL in WI-38 fibroblasts exposed to SSC for 4 and 7 days, compared with N and SS. These results are representative of 12 independent experiments. G, Percentage of apoptotic cells in WI-38 fibroblasts exposed for 7 days to N, SS, or SSC, either alone, with LY294002 (5 μM) or vehicle (DMSO). *, p < 0.0001 vs N; & p < 0.002 vs SSC and SSC-DMSO, representative of eight experiments. H, Immunoblotting analysis. Decreased protein levels of Bcl-xL in WI-38 fibroblasts exposed to SSC concomitantly with LY294002 (5 μM) for 7 days, compared with vehicle (DMSO) and SSC alone. These results are representative of three independent experiments. Two images from the same gel (with corresponding α-tubulin) are shown.

TGF-β1 is appreciated as a central mediator of myofibroblast differentiation (2, 19). Yet, TGF-β1 levels measured in SSC were not different from levels found in normal culture medium (N), which did not induce myofibroblast differentiation (SSC: 0.08 ± 0.04 ng/ml; N: 0.16 ± 0.07 ng/ml; p > 0.05). This suggested that TGF-β1 is not the primary active fibrogenic factor produced by apoptotic EC. Thus, we tested whether motifs present on the C-terminal fragment of perlecain induce myofibroblast differentiation. Fibroblasts exposed for 7 days to SS supplemented with either CS or peptide no. 1 showed increased protein levels of αSMA (Fig. 4E) compared with fibroblasts exposed to serum-free medium for 7 days. The protein levels of αSMA were similar in fibroblasts exposed for 7 days alone to either serum-free medium supplemented with the control peptide or serum-free medium. Coincubation with LY294002 blocked overexpression of αSMA induced by either CS or peptide no. 1. These results suggest that, akin to SSC, the C-terminal fragment of
the domain V of perlecan contains motifs capable of inducing PI3K-dependent myofibroblast differentiation. Yet, CS or peptide no. 1 did not up-regulate desmin protein levels or increase the production of collagen I (data not shown), suggesting that additional mediators present in SSC act coordinately with the fragment of perlecan to induce all features of myofibroblast differentiation.

Systemic sclerosis fibroblasts display increased sensitivity to the antiapoptotic activity of CS and the synthetic peptide

The antiapoptotic activities of SSC, CS, and peptide no. 1 were evaluated on fibroblasts derived from fibrotic skin areas of systemic sclerosis patients or normal controls. Both normal and sclerodermic fibroblasts displayed increased apoptosis after exposure to serum-free medium for 24 h and inhibition of apoptosis in presence of SSC (Fig. 5). In fibroblast cell lines derived from normal controls and exposed to serum-free medium supplemented with CS, the antiapoptotic response developed at a concentration of 500 μg/ml (Fig. 5A), whereas a concentration of 60–125 μg/ml inhibited development of apoptosis in all untransformed fibroblast cell lines derived from systemic sclerosis skin lesion (B). In fibroblasts derived from normal controls, a concentration of ≥10 μg/ml of peptide no. 1 was required to induce an antiapoptotic response (Fig. 5A), whereas resistance to apoptosis developed at a concentration of 100 ng/ml (systemic sclerosis cell lines nos. 1 and 2) to 1 μg/ml (systemic sclerosis cell lines nos. 3 and 4) in fibroblasts derived from sclerodermic skin lesions (B).

Discussion

Systemic sclerosis is a severe and potentially lethal autoimmune disease characterized by early functional and morphologic vascular abnormalities and progressive accumulation of ECM components in the skin and visceral organs of affected individuals. Dysregulation of the molecular control of apoptosis in different cell
types has been implicated in the pathophysiology of various autoimmune diseases, including systemic sclerosis (1). A number of reports suggest that systemic sclerosis is initiated by endothelial apoptosis (1, 5, 6). A possible scenario is that viruses, toxins, or other insults induce endothelial damage, resulting in the production of anti-endothelial Abs, which induce an apoptotic response at the endothelial level (1, 7, 21). Endothelial damage is followed by acquisition of state of resistance to apoptosis in fibroblasts and myofibroblast differentiation, which, on the long term, brings about the characteristic fibrotic changes (1, 22). However, the mechanisms that link these two phenomena (i.e., increased endothelial apoptosis and decreased fibroblast apoptosis) remain ill-defined.
We showed previously that apoptosis of EC triggers the active release of mediators with a robust antiapoptotic activity on vascular smooth muscle cells and EC (8, 9). We suggested that apoptosis contains within its molecular program a paracrine component aimed at fostering repair after an apoptotic insult (8). In the present paper, we evaluated the possibility that, as part of these “built-in” repair pathways, apoptosis of EC triggers the release of mediators active on fibroblasts. Indeed, we found that serum-free medium conditioned by apoptotic EC contains antiapoptotic mediators broadly active on human embryonic pulmonary fibroblasts (WI-38 fibroblasts) and skin fibroblasts derived from normal and systemic sclerosis patients. Serum-free medium conditioned by EC in which development of apoptosis was inhibited (either by caspase inhibition or Bcl-xL overexpression) did not induce resistance to apoptosis in fibroblasts, demonstrating that the antiapoptotic mediators are released by apoptotic EC.

PI3K is a key signaling pathway involved in regulation of cell survival and myofibroblast differentiation (19, 23). Recent reports have suggested that the antiapoptotic phenotype characteristic of sclerodermic fibroblasts develops in association with PI3K.

**FIGURE 6.** Schematic diagram. Apoptosis of EC triggers the release of soluble mediators, which include a C-terminal fragment of perlecan. This, in turn, activates PI3K in fibroblasts leading to resistance to apoptosis, sequentially regulated by modulation of Bim-EL and Bcl-xL protein levels, and to myofibroblast differentiation. However, chronic production of these mediators could lead to fibrosis.
activation and increased phosphorylation of Akt at sites of sclerodermic skin lesions (1). In keeping with a potentially important role for endothelial apoptosis in activating fibrogenic pathways, our results show that mediators produced by apoptotic EC induce resistance to apoptosis in fibroblasts largely through PI3K-dependent mechanisms. We found increased phosphorylation of Akt in fibroblasts exposed to serum-free medium conditioned by apoptotic EC. Inhibition of PI3K activation in fibroblasts with LY294002 blocked the antiapoptotic activity of mediators produced by apoptotic EC. Resistance to apoptosis in fibroblasts was found to be sequentially regulated by decreased Bim-EL protein levels followed by increased Bcl-xL protein levels, both occurring downstream of PI3K activation.

We showed previously that a C-terminal fragment of the domain V of perlecan, which comprises an EGF motif and an anchoring site for CS, is one of the bioactive mediator(s) produced by apoptotic EC (8). Our present results demonstrate that the molecular pattern of resistance to apoptosis induced in fibroblasts by mediators produced by apoptotic EC can be largely reproduced by motifs present on the C-terminal fragment of perlecan. CS and a synthetic peptide containing the last EGF motif of the domain V of perlecan induced PI3K-dependent resistance to apoptosis in fibroblasts and prevented Bim-EL up-regulation. CS also induced long-term PI3K-dependent resistance to apoptosis in fibroblasts in association with increased Bcl-xL protein levels.

Because fibrosis develops through the coordinate actions of increased resistance to apoptosis in fibroblasts and sustained myofibroblast differentiation (2), we evaluated whether long-term exposure of fibroblasts to mediators produced by apoptotic EC induces phenotypic changes characteristic of myofibroblast differentiation. Fibroblasts exposed to medium conditioned by apoptotic EC presented various characteristics of myofibroblast differentiation. Fibroblasts exposed to medium conditioned by apoptotic EC presented various characteristics of myofibroblast differentiation. Fibroblasts exposed to medium conditioned by apoptotic EC prevented Bim-EL up-regulation. CS also induced long-term PI3K-dependent resistance to apoptosis in fibroblasts in association with increased Bcl-xL protein levels.

To test further the potential clinical importance of these mediators on fibrogenesis, we evaluated whether systemic sclerosis fibroblasts display increased sensitivity to the antiapoptotic activity of CS and the synthetic peptide. The antiapoptotic response developed in normal fibroblasts at a concentration of CS four times higher or more than that needed to inhibit apoptosis in fibroblasts derived from scleroderemic patients (24). Motifs present on the C-terminal fragment of perlecan were found to induce PI3K-dependent increased expression of αSMA, the most significant marker of myofibroblastic cells (3). Yet, these motifs did not induce desmin overexpression or increased production of collagen I, suggesting that mediators released by apoptotic EC and yet uncharacterized act coordinately with the C-terminal fragment of perlecan to promote complete myofibroblast differentiation.

To further the potential clinical importance of these mediators on fibrogenesis, we evaluated whether systemic sclerosis fibroblasts display increased sensitivity to the antiapoptotic activity of CS and the synthetic peptide. The antiapoptotic response developed in normal fibroblasts at a concentration of CS four times higher or more than that needed to inhibit apoptosis in fibroblasts derived from scleroderemic skin lesions. This increased sensitivity was even more pronounced for the synthetic peptide harboring the EGF motif, which induced an antiapoptotic response in normal fibroblasts at a concentration 10- to 1000-fold higher than that needed to inhibit apoptosis in systemic sclerosis fibroblasts.

Akin to involution of structures during development, tissues and vessels are reshaped after injury through finely regulated processes that control proliferation, differentiation, and apoptosis (25). These processes need to be dynamically coordinated so that cells that may gain a survival advantage at initiation of repair, such as fibroblasts, myofibroblasts, and smooth muscle cells, are cleared by apoptosis once healing is completed (2, 4). In the past decade, apoptosis research has largely focused on deciphering the intracellular pathways regulating commitment and execution of apoptosis in various cell types (25, 26). The paracrine consequence of apoptosis within a given environment has received much less attention. The present results and past work from our group (8, 9) suggest that apoptosis initiates a complex and finely regulated paracrine response aimed at fostering repair at sites of injury. Apoptosis of EC triggers caspase-dependent release of novel mediators, which include a C-terminal fragment of perlecan. There is no caspase cleavage site in the C-terminal region of the domain V of perlecan, suggesting that an endoprotease(s), as yet uncharacterized and activated downstream of caspases in apoptotic EC, is responsible for the proteolysis of perlecan. Generation of bioactive mediators by apoptotic cells through proteolysis of ECM components represents an “energy-efficient” solution for triggering repair pathways, because the apoptotic proteolytic machinery would serve concomitantly at the execution of apoptosis and release of cryptic bioactive factors. The ECM is a known reservoir of growth factors and cryptic bioactive factors (27–29), whereas cues from cell-matrix interactions are key to maintenance of cell survival (15, 30). Our results suggest that apoptosis of EC represents a novel mechanism for induction of ECM proteolysis and production of cryptic bioactive factors activating normal repair mechanisms such as acquisition of resistance to apoptosis in fibroblasts and myofibroblast differentiation. These results also suggest that persistent activation of endothelial apoptosis presents an unrecognized potential toward initiation of fibrosis. Sustained production of mediators by EC chronically exposed to apoptotic stimuli or with an intrinsic tendency toward apoptosis could perturb the homeostatic regulation of apoptosis in fibroblasts and myofibroblasts. Hence, a chronic increase in endothelial apoptosis and/or increased sensitivity of fibroblasts to mediators produced by apoptotic EC could form the basis of a maladaptive healing process characteristic of fibrotic diseases such as systemic sclerosis (Fig. 6).

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Disclosures
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