Perlecan Proteolysis Induces an α2β1 Integron- and Src Family Kinase-dependent Anti-apoptotic Pathway in Fibroblasts in the Absence of Focal Adhesion Kinase Activation

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Dysregulation of apoptosis in endothelial cells (EC) and fibroblasts contributes to fibrosis. We have shown previously that apoptosis of EC triggers the proteolysis of extracellular matrix components and the release of a C-terminal fragment of perlecan, which in turn inhibits apoptosis of fibroblasts. Here we have defined the receptors and pathways implicated in this anti-apoptotic response in fibroblasts. Neutralizing α2β1 integrin activity in fibroblasts exposed to either medium conditioned by apoptotic EC (SSC) or a recombinant perlecan C-terminal fragment (LG3) prevented resistance to apoptosis and is associated with decreased levels of Akt phosphorylation. Co-incubation of fibroblasts for 24 h with SSC or LG3 in the presence of PP2 (AG1879), a biochemical inhibitor of Src family kinases (SFKs) and focal adhesion kinase, showed a significantly decreased anti-apoptotic response. However, focal adhesion kinase gene silencing with RNA interference did not inhibit the anti-apoptotic response in fibroblasts. Src phosphorylation was increased in fibroblasts exposed to SSC, and transfection of fibroblasts with constitutively active Src mutants induced an anti-apoptotic response that was not further increased by SSC. Also, Src−/−/Fyn−/− fibroblasts failed to mount an anti-apoptotic response in presence of SSC for 24 h but developed a complete anti-apoptotic response when exposed to SSC for 7 days. These results suggest that extracellular matrix fragments produced by apoptotic EC initiate a state of resistance to apoptosis in fibroblasts via an α2β1 integrin/SFK (Src and Fyn)/phosphatidylinositol 3-kinase (PI3K)-dependent pathway. In the long term, additional SFK members are recruited for sustaining the anti-apoptotic response, which could play crucial roles in abnormal fibrogenic healing.

Fibrotic diseases are characterized by increased deposition of extracellular matrix (ECM) components associated with persistent accumulation of fibroblasts and myofibroblasts, the latter being a specialized type of fibroblast implicated in repair processes at sites of tissue injury (1–4). The mechanisms leading to fibrosis include a chronic state of resistance to apoptosis in fibroblasts and myofibroblasts during the resolution phase of the repair response, thus preventing clearance of unneeded cells (2, 5, 6). The persistent deposition of ECM components favors the deformation of the injured tissue leading to progressive loss of function (2, 7). Activation of the pro-survival phosphatidylinositol 3-kinase (PI3K) pathway and up-regulation of the anti-apoptotic protein Bcl-xL have been documented within fibroblasts or myofibroblasts in various fibrogenic models and conditions, including systemic sclerosis, chronic transplant vasculopathy, and bleomycin-induced lung fibrosis (8–10). Inhibition of PI3K activation or use of Bcl-xL antisense oligonucleotides in animal models of fibrosis were shown to prevent resistance to apoptosis in fibroblasts and myofibroblasts and attenuated fibrogenic changes (9–11). Hence, fibroblasts play an important role in tissue repair, but their survival has to be tightly regulated to avoid maladaptive and pathogenic healing processes.

In various fibrotic diseases, increased apoptosis of endothelial cells (EC) has been shown to precede recruitment of fibroblasts (12–16). Our group showed that apoptosis of EC induces ECM proteolysis, leading to the production of novel cryptic fibrogenic factors (17, 18). In turn, these ECM fragments inhibit the apoptosis of fibroblasts, enhance production of collagen I by fibroblasts, and favor myofibroblast differentiation (18). These anti-apoptotic and potentially fibrogenic mediators are released specifically by apoptotic cells, because inhibition of apoptosis in EC with either pancaspase inhibition or overexp
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pression of Bcl-x<sub>L</sub> prevents the release of anti-apoptotic factors (17, 18). Hence, we proposed that sustained EC apoptosis represents a novel mechanism for initiating and/or perpetuating fibrogenic changes (13, 18).

We identified a C-terminal fragment of perlecan as one of the ECM-derived fibrogenic mediators produced by apoptotic EC (17, 18). Perlecan is a large modular proteoglycan present in the basement membrane and produced by cellular constituents of the vessel wall, including EC (19, 20). This C-terminal fragment inhibits the apoptosis of fibroblasts through PI3K-dependent pathways leading to down-regulation of the pro-apoptotic protein Bim-EL and up-regulation of the anti-apoptotic protein Bcl-x<sub>L</sub> (18). Yet, the cell surface receptors and signaling pathways that initiate this anti-apoptotic response upstream of PI3K activation remain to be defined.

We have shown previously (21) that the laminin G motif LG3 present on the C-terminal fragment of perlecan interacts with α2β1 integrin receptors on EC and thus induces a potent angiostatic response. Integrins are heterodimeric transmembrane receptors that exert a stringent control on cell survival, proliferation, migration, and adhesion. Integrin-dependent PI3K activation has been classically attributed to the binding of activated Src to the p85 subunit of PI3K or from the autophosphorylation of focal adhesion kinase (FAK) on Tyr<sup>397</sup> (22–24).

Recent results suggest that activation of PI3K upon integrin engagement can also occur through other Src-dependent and FAK-independent pathways (25) or via the membrane-proximal part of the β1 integrin subunit in a FAK- and Src-independent manner (26). Here we tested the hypothesis that ECM fragments produced by apoptotic EC (and more specifically the LG3 motif of perlecan) interact with integrin receptors on fibroblasts, thus initiating integrin-dependent signaling events leading to the activation of a PI3K-dependent anti-apoptotic and potentially fibrogenic phenotype in fibroblasts.

MATERIALS AND METHODS

Cell Lines—Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA), grown in endothelial cell basal medium (Clonetics) and used at passages 2–4. WI-38 human fibroblasts from normal embryonic lung tissue were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), grown in fibroblast basal medium (Cambrex; Walkersville, MD) supplemented with 10% inactivated fetal bovine serum (Medicorp; Montreal, Quebec, Canada) and used at passages 2–17.

Fibroblasts genetically deficient for Src (Src<sup>−/−</sup>) and/or Fyn (Fyn<sup>−/−</sup>) were isolated from mouse embryo fibroblasts homozygous for disruption of the Src and/or Fyn genes and immortalized with a large T antigen (27). Cells were kindly provided by Dr. Sheila M. Thomas (Cancer Biology Program, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA). Mouse fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Wisent, Inc.; Saint-Bruno, Quebec, Canada) supplemented with 10% inactivated fetal bovine serum.

Production of Recombinant C-terminal Fragment of Perlecan—Production of a recombinant perlecan fragment containing the C-terminal laminin G-like motif LG3 was performed as described previously (28). In brief, we cloned and purified the LG3 C-terminal fragment of the domain V of perlecan corresponding to amino acids 4197–4391. RNA was extracted from HUVECs as described previously (17) and was amplified by reverse transcription-PCR using the following primers for the LG3 motif: sense, 5′-GCGGCAGCGCATGCCCCT-3′; reverse, 5′-GCGCCGCCTACGAGGG-3′. The amplified cDNA sequence was cloned at the NotI restriction site in the pCEP4 plasmid vector modified by the addition of a Kosak sequence and a triple repeat hemagglutinin sequence (kind gift from Dr. Richard Bertrand, University of Montreal, Quebec, Canada). Stable transformants were obtained in Namalwa cells (ATCC). Expression of the transgene was confirmed by immunoblotting using an anti-hemagglutinin antibody (Roche Diagnostics). For production of recombinant protein, stable transformants were harvested and proteins were extracted as described above and purified on an anti-hemagglutinin affinity matrix (Roche Diagnostics) according to the protocol provided by the manufacturer. A second recombinant human LG3 was also produced in 293-EBNA cells as described previously (29).

Screening for Apoptosis with Fluorescence Microscopy—Fluorescence microscopy of unfixed/unpermeabilized adherent cells stained with Hoechst 33342 (HT) and propidium iodide was used as described in our previous work (17, 18, 30). In brief, cells were grown to confluence in 24-well polystyrene culture plates (Corning Incorporated, Corning, NY). HT (1 μg/ml) was added for 10 min at 37 °C, and the cells were washed with phosphate-buffered saline. Propidium iodide 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 displayed normal nuclear and cytoplasmic morphology and stained blue. Early apoptotic cells were characterized by cell shrinkage, nuclear condensation, and preservation of plasma membrane integrity. Chromatin condensation was associated with enhanced fluorescence for HT (bright blue), whereas preservation of cell membrane integrity precluded propidium iodide staining. Late apoptotic cells (also called secondary necrosis) were characterized by the presence of characteristic apoptotic nuclear changes and loss of cell membrane integrity associated with propidium iodide staining. Primary necrotic cells were 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 (17, 18, 30). A polyclonal antibody directed against the C-terminal end of perlecan was synthesized in New Zealand White rabbits by Sigma Genosys using a synthetic human C-terminal perlecan peptide as immunogen (amino acids 4351–4391 (5′-GTTGCVKNVLH- SARGPAPPQPLQDLHRAQAGANTRPPCPS-3′)). In brief, two rabbits were injected with a Keyhole Limpet hemocyanin conjugate of the synthetic peptide. The first injection (day 1) was done with 200 μg of conjugate/rabbit and was followed with five booster shots of 100 μg of conjugate at an interval of 2
weeks. One week following the last injection, the rabbits were killed and exsanguinated. Preimmune serum was also prepared from the blood and used as an internal control. An enzyme-linked immunosorbent assay was performed to confirm the affinity of test bleeds against the C-terminal perlecan peptide. The other antibodies used for Western blotting were anti-Bcl-xL (Pharmingen), anti-Bim-EL (Calbiochem), anti-phospho-FAK (Tyr397) (Calbiochem), anti-FAK (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-Src (Tyr 418) (BIOSOURCE, Camarillo, CA), anti-phospho-Akt (Ser473) and anti-Akt (Cell Signaling Technology Inc., Beverly, MA).

After initial probing, all membranes were stripped (2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.7) at 50 °C for 30 min with gentle shaking and reprobed with an anti-α-tubulin monoclonal antibody (Oncogene; Boston, MA) as a control for protein loading. Densitometric analyses were performed using Alphalager, version 3.2 (Alpha Innotech Corporation, San Leandro, CA).

Mammalian Expression Vectors and Transfections—The plasmid expressing a constitutively active Src mutant (Src cDNA activated in pUSEamp-Y529F mutation) was from Upstate Biotechnology (Lake Placid, NY), and the expression plasmid encoding constitutively active Src tagged with GFP was a kind gift from Dr. M. Bouvier, University of Montreal. A control GFP plasmid (pEGFP-C1) was purchased from Clontech (Mountain View, CA). WI-38 fibroblasts were transfected with Lipofectin (Invitrogen) according to the protocol provided by the manufacturer. Briefly, WI-38 cells were plated onto 24-well plates at 20,000 cells/well and transfected for 8 h with 1 μg of plasmid using 5 μl of Lipofectin. After 60 h, the cells were placed in experimental conditions for 24 h followed by evaluation of apoptosis with fluorescence microscopy.

Immunoprecipitation of Focal Adhesion Kinase—Five hundred μg of the cell lysate were incubated with 2 μg of FAK antibody at 4 °C for 2 h in 250 μl of immunoprecipitation buffer (1% Triton X-100, 250 mM NaCl, 20 mM Tris, 2 mM EDTA, 2 mM EGTA, 40 μM leupeptin, 40 μM pepstatin, 0.4 mM phenylmethylsulfonyl fluoride, 4 mM Na3VO4, 10 mM Na2HPO4, 50 mM NaF, pH 7.4). Twenty-five μl of protein A/G-agarose were added and incubated for 2 h at 4 °C. The tubes were centrifuged, supernatants were harvested, and pellets were washed four times in 200 μl of immunoprecipitation buffer. A fifth wash was done with a Tris buffer at pH 7 (50 mM Tris, 0.01% SDS). The pellets were dissolved in Laemmli reducing buffer to proceed to Western blot.

RNA Interference—WI-38 fibroblasts were plated onto 6-well and 24-well plates at 100,000 and 20,000 cells/well, respectively. After 20 h, the cells were transfected with double-stranded RNA-DNA hybrids at a final concentration of 200 nM annealed oligo using Oligofectamine (Invitrogen). After 45 h of transfection, the cells were placed in experimental conditions for 24 h followed by evaluation of apoptosis with fluorescence microscopy or evaluation of FAK protein levels by Western blotting. Oligonucleotides were obtained from Pharmaco Research (Lafayette, CO). FAK sense was 5′-(AACCACCGUGGCGCUGUUAUUAU) 3′, FAK antisense was 5′-(AAGCUAAACUGGGCCGCGUGG) 3′, control sense was 5′-(GACGUGGGACUGAAGGGGU) 3′, and control antisense was 5′-(AAGCUAAACUGGGCCGCGUGG) 3′.

**FIGURE 1.** Anti-apoptotic response induced by the third laminin G motif LG3 of the C-terminal fragment of perlecan. A, left, percentage of apoptotic cells in WI-38 fibroblasts exposed for 24 h to normal medium (N), serum-free medium (SS), and serum-free medium conditioned by apoptotic HUVECs (SSC) either alone, with PP2 (10 μM) or control (PP3) (*, p < 0.05) versus SS (&, p < 0.04) versus SSC and PP3; n = 4. Right, percentage of apoptotic cells in WI-38 fibroblasts exposed for 7 days to N, SS, and SSC either alone, with PP2, or control (PP3) (*, p < 0.0008) versus SS (&, p < 0.0006) versus SSC and PP3; n = 18. Lower panel, representative micrographs of WI-38 fibroblasts exposed for 24 h to N, SS, and SSC either alone, with PP2, or control (PP3) followed by staining with Hoechst 33342 and propidium iodide (magnification 200x). B, Western blot. C, percentage of apoptotic cells in WI-38 fibroblasts exposed for 24 h to N, SS, and SS supplemented with the LG3 recombinant peptide (1 μg/ml) either alone, with PP2, or inactive control (PP3) (*, p < 0.05) versus SS (&, p < 0.0003) versus LG3 and LG3 + PP3; n = 7.
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A. 4 hours

5'- (ACCCCUUCAGUCCACGUC)d(TT)-3' as previously reported (31).

Reagents—PP2 and PP3 were purchased from Calbiochem. Anti-α2β1 blocking antibodies were purchased from Chemicon International (Temecula, CA), and mouse IgG1 was from R & D systems (Minneapolis, MN). All other reagents were from Sigma.

Statistical Analysis—Results were expressed as mean ± S.E. Data were analyzed using Student's t test or analysis of variance, as appropriate. A p < 0.05 was considered significant for all tests.

RESULTS

Anti-apoptotic Activity of ECM Fragments Produced by Apoptotic Endothelial Cells—Serum-free medium conditioned by apoptotic EC (SSC) was generated as we described previously (17, 18, 30). In brief, apoptosis of HUVECs was induced with serum starvation for 4 h to generate SSC. We have shown previously (18) that fibroblasts exposed to SSC develop an anti-apoptotic response, whereas exposure to serum-free medium conditioned by non-apoptotic HUVECs does not induce an
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To further prove that this C-terminal fragment of perlecain is released by apoptotic EC, we evaluated by Western blotting the protein levels of this perlecain fragment in SSC and in medium conditioned by non-apoptotic EC (SSC-Z-VAD). We generated serum-free medium conditioned by non-apoptotic EC as described previously (18). In brief, HUVECs were exposed to Z-VAD-fmk (100 μM) or vehicle (dimethyl sulfoxide, Me2SO) for 2 h. Z-VAD-fmk and vehicle were then removed, and HUVECs were washed and serum-starved for 4 h. We have shown previously (17) that pre-incubation with Z-VAD-fmk significantly decreased the percentage of apoptotic EC after 4 h of serum starvation, whereas pre-incubation with Me2SO did not. Using an antibody directed against the C-terminal end of perlecain, we found that the level of this perlecain fragment is increased in SSC as compared with SSC-Z-VAD or SS (Fig. 1B). No band of high molecular weight was found in SSC (Fig. 1B), suggesting that uncleaved perlecain is not released in conditioned media, further supporting the contention that EC apoptosis activates ECM proteolysis. Fibroblasts exposed for 24 h to SS supplemented with a recombinant peptide homologous to the C-terminal laminin G motif of perlecain LG3 showed increased resistance to apoptosis as compared with SS alone (Fig. 1C). A second recombinant LG3, tagged with a hemagglutinin motif, also induced resistance to apoptosis in fibroblasts in a dose-dependent manner (data not shown). PP2 blocked the anti-apoptotic response induced by the LG3 recombinant peptide in fibroblasts, whereas PP3 did not (Fig. 1C), thus suggesting that LG3 and SSC initiate similar anti-apoptotic pathways in fibroblasts.

Fibroblasts exposed to the mediators released by apoptotic EC develop a state of resistance to apoptosis dependent on Bim-EL down-regulation and Bcl-xL up-regulation, both occurring downstream of PI3K activation (18). We found that phosphorylation of Akt was reduced in fibroblasts exposed to SSC in the presence of PP2 as compared with SSC alone and PP3 (Fig. 2A). This result suggests that a member of SFK and/or FAK is/are activated upstream of PI3K. In support of this contention, modulations of Bim-EL (Fig. 2B) and Bcl-xL (Fig. 2C) protein

ant-apoptotic phenotype in fibroblasts. Fibroblasts exposed to SSC for 24 h in the presence of PP2, an Src family kinase (SFK) and FAK inhibitor, showed a complete blockade of the anti-apoptotic response (Fig. 1A). PP3, the inactive control, did not modulate the anti-apoptotic response of fibroblasts exposed to SSC (Fig. 1A). PP2 alone did not induce or increase development of apoptosis in fibroblasts exposed to serum-free medium (SS) (apoptosis of fibroblasts exposed to serum-free medium in the presence of PP2 or PP3 for 24 h; % of apoptotic cells SS + PP2 = 2.32 ± 0.59%, SS + PP3 = 1.33 ± 0.81%; p > 0.34; n = 8). PP2 also significantly reduced the anti-apoptotic response mounted by fibroblasts exposed to SSC for 7 days, although incompletely (Fig. 1A).

We showed in previous work that a C-terminal fragment of perlecain is found in the biologically active fraction of SSC (18).
levels were attenuated in fibroblasts exposed to SSC with PP2 for 24 h and 7 days but were conserved in the presence of PP3. These results suggest that mediators produced by apoptotic EC inhibit apoptosis of fibroblasts through activation of SFK and/or FAK-dependent pathways.

Activation of SFK Is Required for Transduction of PI3K-dependent Anti-apoptotic Signals in Fibroblasts—We went on to determine which SFK in fibroblasts are implicated in the transduction of anti-apoptotic signals initiated by mediators produced by apoptotic EC. Fibroblasts exposed to SSC showed increased levels of Src phosphorylation (Fig. 3A). Concomitant exposure to PP2 decreased Src phosphorylation as compared with SSC alone or SSC+/H11001PP3 (Fig. 3A). To evaluate the functional importance of Src in our system, fibroblasts were transfected with a constitutively active Src-mutant, GFP-tagged constitutively active Src or GFP control. Fibroblasts untransfected or transfected with the GFP plasmid developed an apoptotic response when exposed to serum-free medium for 24 h as compared with normal medium (data not shown). Wild-type mouse fibroblasts showed a similar pattern of resistance to apoptosis as WI-38 fibroblasts when exposed to SSC (Fig. 3C). We then evaluated whether mediators produced by apoptotic EC block apoptosis of Src−/− and/or Fyn−/− fibroblasts induced by serum starvation, Src−/− and Fyn−/− fibroblasts incubated for 24 h in the presence of SSC showed significantly less apoptosis as compared with SS (Fig. 3C). However, Src−/−Fyn−/− fibroblasts failed to develop an anti-apoptotic response in the presence of SSC (Fig. 3C). These results suggest that the coordinate activation of Src and Fyn in fibroblasts exposed to SSC is needed for the transduction of anti-apoptotic signals initiated by SSC.

We also evaluated the importance of SFK on the long term development of resistance to apoptosis. In keeping with a partial blockade of the anti-apoptotic response in WI-38 fibroblasts by PP2 at 7 days (Fig. 1A), Src−/−Fyn−/− fibroblasts...
developed an anti-apoptotic response in the presence of SSC for 7 days (Fig. 3C). These results suggest that, in the long term, additional mechanisms kick in to perpetuate the anti-apoptotic phenotype.

**FAK Activity Is Dispensable for PI3K Activation and Development of an Anti-apoptotic Response**—We then evaluated whether FAK activation is necessary for transduction of the anti-apoptotic signals in fibroblasts exposed to mediators produced by apoptotic EC. Phosphorylation of FAK at Tyr397 was not modulated in fibroblasts exposed to SSC, SSC/H11001PP2, or SSC/H11001PP3 for 2, 15, or 30 min (data not shown), whereas increased Src phosphorylation was already evident after exposure to SSC for 30 min (Fig. 3A). These results suggest that FAK is dispensable for the anti-apoptotic activity of SSC on fibroblasts. To further prove this contention, we evaluated the consequences of FAK gene silencing on the development of an anti-apoptotic response in fibroblasts exposed to SSC. Small interfering RNA for FAK effectively decreased FAK protein levels (Fig. 4A). However, FAK silencing did not alter the anti-apoptotic response normally induced by SSC in fibroblasts (Fig. 4B). These results suggest that mediators released by apoptotic EC inhibit apoptosis of fibroblasts through an SFK- and PI3K-dependent pathway, independently of FAK activation.

**α2β1 Integrins Are Activated Upstream of Src and PI3K**—We then sought to define the cell surface receptor activated upstream of SFK in fibroblasts exposed to ECM-derived mediators produced by apoptotic EC and, more specifically, the LG3 motif of the C-terminal fragment of perlecan (17, 18). As α2β1 integrins have been implicated in the angiostatic activity of LG3 (21), we went on to test whether α2β1 integrins are required for transduction of anti-apoptotic signals in fibroblasts. Fibroblasts were pretreated for 30 min in serum-free medium with a function-blocking antibody directed against α2β1 integrin (10 μg/ml) or control mouse IgG1 (10 μg/ml) followed by co-incubation with SSC for 24 h. Neutralizing antibodies against α2β1 integrin antibody abolished the anti-apoptotic activity normally induced by SSC in fibroblasts, whereas the control isotype-matched antibody did not (Fig. 5A). The neutralizing anti-α2β1 integrin antibody also significantly decreased the anti-apoptotic response of fibroblasts exposed to recombinant LG3 (Fig. 5B). Fibroblasts exposed to SSC in the presence of the

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**FIGURE 5.** α2β1 integrin activation leads to a PI3K-dependent anti-apoptotic phenotype in fibroblasts. A, percentage of apoptotic cells in WI-38 fibroblasts exposed for 24 h to normal medium (N), SS, or SSC, either alone, with a function-blocking antibody against α2β1 integrin (10 μg/ml), or control mouse IgG1 (*, p < 0.05) versus SS (&, p < 0.05) versus SSC and IgG; n = 13. B, percentage of apoptotic cells in WI-38 fibroblasts exposed for 24 h to N, SS, or SS + recombinant LG3 (1 μg/ml), either alone, with a function-blocking antibody against α2β1 integrin (10 μg/ml), or control mouse IgG1, (*, p < 0.05) versus SS (&, p < 0.03) versus SSC and IgG; n = 14. C, immunoblotting analysis. Decreased protein levels of AKT-PO4 in WI-38 fibroblasts exposed to SSC concomitantly with α2β1 integrin-blocking antibody for 4 h, as compared with control (mouse IgG1) and SSC alone. These results are representative of four independent experiments. Autoradiographs were quantified by densitometric scanning for phosphorylated-AKT relative to the amount of total AKT; SSC + α2β1 was different from SSC and SSC + IgG (*, p < 0.05).
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![Diagram of Apoptotic EC and Fibroblast Survival](Image)

α2β1 antibody showed decreased Akt phosphorylation as compared with fibroblasts exposed to SSC alone or SSC plus the isotype-matched control antibody (Fig. 5C). These results suggest that mediators produced by apoptotic EC, and more specifically the LG3 motif of cleaved perlecan, interact with α2β1 integrins leading to a SFK/PI3K-dependent anti-apoptotic phenotype in fibroblasts.

**DISCUSSION**

Dysregulation of the molecular control of apoptosis is an important contributor to fibrogenesis (2, 7, 18). In various fibrotic diseases, apoptosis of EC is an early event and precedes recruitment of fibroblasts, myofibroblast differentiation, and sustained production of ECM components (8, 12–16). Chronic accumulation of fibroblasts and myofibroblasts is a hallmark of fibrosis and appears to be due, at least in part, to the development of a state of long term resistance to apoptosis in fibroblasts (2, 32, 33). We have shown previously (17, 18) that apoptosis of EC triggers the proteolysis of ECM components and the release of cryptic bioactive factors, which include a C-terminal fragment of perlecan. The LG3 domain of the C-terminal fragment of perlecan interacts with α2β1 integrin receptors initially activating Src- and Fyn-dependent pathways leading to activation of PI3K and repression of apoptosis. In the long term, additional members of the SFK are recruited to modulate Bcl-xL protein levels through PI3K-dependent and possibly -independent pathways implicated in the perpetuation of this anti-apoptotic phenotype. Sustained production of these cryptic factors would prevent apoptosis-mediated clearance of fibroblasts at sites of injury, thus potentially fostering a fibrogenic response.

ECM fragments released in association with EC apoptosis interact with α2β1 integrins on fibroblasts to induce a PI3K-dependent anti-apoptotic phenotype.

Most integrins signal predominantly through the recruitment and activation of Src-family kinases (SFK) and FAK (24, 26). PI3K is a downstream target of integrin-FAK/SFK pathways and can be activated through various and non-mutually exclusive pathways (34–36). Upon integrin ligation, activation of PI3K can result from the binding of the Src homology 3 domain of Src with a proline-rich region within the p85 subunit of PI3K or through interactions between p85 and the p85 homology 3 domain of Src with a proline-rich region within the p85 subunit of PI3K or through interactions between p85 and the p85 homology 3 domain of Src with a proline-rich region within the p85 subunit of PI3K (24, 26). PI3K is a downstream target of integrin-FAK/SFK pathways and can be activated through various and non-mutually exclusive pathways (34–36). Upon integrin ligation, activation of PI3K can result from the binding of the Src homology 3 domain of Src with a proline-rich region within the p85 subunit of PI3K. Integrin-dependent FAK autophosphorylation on Tyr397 may lead to the recruitment of Src homology 2 domain containing signaling proteins such as PI3K (22). Recent results suggest that the membrane proximal part of the β1 integrin subunit may also induce Akt phosphorylation independently of FAK and Src (26, 38). Thus, the interplay between FAK, SFK, and PI3K was expected to be of potential interest in our experimental system.

Fibroblasts exposed to SSC in the presence of PP2 (or AG1879), a biochemical inhibitor of FAK and SFK, for 24 h and up to 7 days showed, respectively, complete and partial inhibition of the anti-apoptotic response and decreased Akt phosphorylation. Two recombinant peptides containing the C-terminal laminin G motif LG3 of domain V of perlecan induced...
resistance to apoptosis in fibroblasts, and again resistance to apoptosis was inhibited in presence of PP2.

Because PP2 may potentially block both FAK and SFK, we set out to characterize which pathway is indispensable for transduction of anti-apoptotic signals initiated by ECM fragments released by apoptotic EC. No changes in FAK phosphorylation on Tyr397 were found in fibroblasts exposed to SSC. Also, FAK gene silencing with RNA interference, which resulted in a profound down-regulation of FAK protein levels in fibroblasts, did not block the anti-apoptotic activity of mediators produced by apoptotic EC. These results demonstrated that FAK is dispensable for the transduction of anti-apoptotic signals in this system and suggested that SFK may play a pivotal role. In support of this contention, we found that Src is phosphorylated in fibroblasts exposed to SSC and that concomitant exposure of fibroblasts to SSC and PP2 blocked Src phosphorylation. Also, fibroblasts transfected with constitutively active Src mutants developed an anti-apoptotic phenotype. Exposure to SSC did not further increase this anti-apoptotic response, suggesting that SSC and constitutively active Src activate similar downstream anti-apoptotic pathways. Finally, we found that Src−/−/Fyn−/− double knock-out fibroblasts do not mount an anti-apoptotic response when exposed to SSC for 24 h, whereas fibroblasts from wild-type controls and Src−/− or Fyn−/− single knock-out do. Src−/−Fyn−/− double knock-out fibroblasts did not show increased AKT phosphorylation after exposure to SSC (data not shown). These results demonstrate that the early development of resistance to apoptosis in fibroblasts exposed to mediators produced by apoptotic EC relies on the coordinate activation of Src and Fyn, leading to PI3K activation and downstream activation of an anti-apoptotic response.

Long term exposure of fibroblasts to SSC, however, was associated with the development of a chronic state of resistance to apoptosis largely independent of Src or Fyn. Double knock-out fibroblasts developed an anti-apoptotic response of a magnitude similar to wild-type controls or Src−/− or Fyn−/− single knock-out fibroblasts when exposed to SSC for 7 days. In keeping with these results, we found that WI-38 fibroblasts exposed to SSC for 7 days in the presence of PP2 showed a decreased (but still present) anti-apoptotic response. PP2 may inhibit members of the SFK other than Src and Fyn (39), which may explain the apparent discrepancy between the decreased anti-apoptotic response in WI-38 fibroblasts exposed to PP2 and the complete anti-apoptotic response developed by Src−/−Fyn−/− double knock-out fibroblasts. These results suggest that, in the long term, additional SFK members and pathways are recruited for sustaining the anti-apoptotic response, which could play crucial roles in fibrogenesis.

Taken together, these results suggest that ECM fragments released during EC apoptosis, which include a C-terminal fragment of domain V of perlecian, induce resistance to apoptosis in fibroblasts via an α2β1 integrin/SFK (Src and Fyn)/PI3K-dependent pathway (Fig. 6). The precise mechanisms responsible for activation of Src and Fyn by α2β1 integrins will be further delineated in future experiments. Direct interactions between the cytoplasmic tail of β1 integrins and Src and between Fyn and the α subunit of various integrins have been characterized (24, 40) and could be operational in our system. Also, the precise characterization of the SFK members implicated in the development of a chronic state of resistance to apoptosis in fibroblasts, and thus possibly in maladaptive healing, will also be the scope of future studies.

Competition of cell/matrix interactions by soluble peptide fragments derived from the proteolysis of ECM components (such as fibronectin, tenascin-C, and SPARC (“secreted protein acidic and rich in cysteine”)) have been shown to interfere with integrin-dependent signaling and stimulate focal adhesion disassembly and cell death (41–44). Our results demonstrate that ECM fragments produced by apoptotic EC may also interact with integrin receptors to initiate potent anti-apoptotic pathways rather than pro-death signals. Fibroblasts play a beneficial role in the reconstruction of tissue architecture after injury. Increased resistance to apoptosis in fibroblasts at sites of EC injury could facilitate repair and likely represents a normal component of the healing response (2). Yet, a chronic state of resistance to apoptosis in fibroblasts has been implicated in inappropriate accumulation of fibroblasts at sites of injury and forms the basis of fibrotic diseases (2, 7, 13, 18, 32). The present results suggest that various members of the SFK cooperate to initiate and perpetuate an anti-apoptotic response in fibroblasts and suggest new targets to modulate regulation of fibroblast apoptosis at sites of pathological repair.

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