FAK blunts adenosine-homocysteine-induced endothelial cell apoptosis: requirement for PI 3-kinase

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FAK blunts adenosine-homocysteine-induced endothelial cell apoptosis: requirement for PI 3-kinase. Am J Physiol Lung Cell Mol Physiol 282: L1135–L1142, 2002. First published January 4, 2002; 10.1152/ajplung.00174.2001.—Treatment of cultured bovine pulmonary endothelial cells (BPAEC) with adenosine (Ado) alone or in combination with homocysteine (Hc) leads to disruption of focal adhesion complexes, caspase-dependent degradation of components of focal adhesion complexes, and subsequent apoptosis. Endothelial cells transiently overexpressing paxillin or p130Cas cDNAs underwent Ado-Hc-induced apoptosis to an extent similar to that of cells transfected with vector alone. However, overexpression of focal adhesion kinase (FAK) cDNA blunted Ado-Hc-induced apoptosis. FAK constructs lacking the central catalytic domain or containing a point mutation, rendering the catalytic domain enzymatically inactive, did not provide protection from apoptosis. Constructs containing a mutation in the major autophosphorylation site (tyrosine-397) similarly did not prevent cell death. A FAK mutant in amino acid 395, deficient in phosphatidylinositol 3-kinase (PI 3-kinase) binding, was not able to blunt apoptosis. Finally, overexpression of FAK did not provide protection from apoptosis in the presence of LY-294002, a PI 3-kinase inhibitor. Taken together, these data suggest that the survival signals mediated by overexpression of FAK in response to Ado-Hc-induced apoptosis require a PI 3-kinase-dependent pathway.

Endothelial cell apoptosis is associated with vascular injury in atherosclerosis (6), hyperoxia-induced lung injury (2), acute respiratory distress syndrome (31), primary pulmonary hypertension (27), and allograft rejection of heart transplant (38). Moreover, the antiangiogenic properties of angiostatin and endostatin are due to their ability to induce endothelial cell apoptosis (12, 15). Thus apoptosis is an important aspect of endothelial cell biology.

We previously showed that extracellular ATP or adenosine (Ado) at 100 μM induces endothelial apoptosis (14, 34). Such a concentration is likely achieved in the local microenvironment, at least transiently, during processes such as degranulation of platelets, cellular necrosis (as might occur during sepsis, tissue injury, ischemia, or rhabdomyolysis), after sympathetic nerve stimulation, and after membrane transporter-mediated release (20). The mechanism of apoptosis induction includes extracellular ATP hydrolysis and cellular uptake of Ado by cells (14). The ability of the methionine metabolite homocysteine (Hc) to potentiate Ado apoptosis, taken together with the similar induction of apoptosis by S-adenosylhomocysteine hydroxylase inhibitors, suggested that the mechanism of ATP- or Ado-induced apoptosis involved S-adenosylhomocysteine hydroxylase inhibition (34).

We previously demonstrated that Ado-Hc-induced apoptosis is accompanied by disruption of focal adhesion complexes (FAC), followed by caspase-dependent degradation of their component proteins, including focal adhesion kinase (FAK), paxillin, and p130Cas (22). FAC consist of proteins that link intracellular actin cytoskeleton to extracellular matrix (ECM) components via cell surface integrins (13, 21). Among the proteins in the complexes are nonreceptor tyrosine kinases such as c-Src and FAK. FAK is a 125-kDa

Apoptosis (programmed cell death) plays a role in normal morphogenesis and homeostasis of tissues (43). The apoptotic process can be initiated by a variety of factors, including various cytokines, loss of adhesion to substratum, or oxidative stress (2, 17, 43). Once initiated, apoptosis programs involve multiple biochemical pathways, including activation of kinases, phosphatases, and proteases, and also altered mitochondrial function (43). The end results of these processes include DNA condensation, blebbing of the plasma membrane, and cytoplasmic shrinkage that ultimately leads to the formation of apoptotic bodies destined for destruction by neighboring cells. Ultimately, whether a cell traverses this pathway to completion is a function of a balance between conflicting pro- and antiapoptotic signals conveyed via a large number of apoptosis-related gene products, including the caspase and Bcl-2 families.

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nonreceptor kinase important in the assembly of FAC and organization of the cytoskeleton (21). On cell adhesion, FAK undergoes autophosphorylation at tyrosine-397 (21). Once phosphorylated, this tyrosine residue serves as a binding site for the SH2 domains of c-Src or phosphatidylinositol 3-kinase (PI 3-kinase) (36). Autophosphorylation of FAK leads to further FAC formation and recruitment of adaptor proteins such as paxillin or p130Cas.

In light of the fact that Ado-Hc treatment causes FAK, paxillin, and p130Cas degradation, we sought to determine whether 1) expression of FAC components would provide endothelial cell survival signals and 2) the downstream signaling pathways were involved in endothelial cell survival. We employed a strategy of analyzing apoptosis in single cells transiently transfected with expression vectors for these proteins. We found that overexpression of FAK, but not paxillin or p130Cas, partially rescues endothelial cells from Ado-Hc-induced apoptosis and that this rescue requires PI 3-kinase activity.

MATERIALS AND METHODS

Cell culture. Bovine pulmonary artery endothelial cells (BPAEC) were obtained via a enzyme-free scraping technique as previously described (14) and maintained in MEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Fungizone). Cells were used at passages 1–10.

Reagents. Ado, dl-homocysteine, and Hoechst-33342 were obtained from Sigma Chemical (St. Louis, MO), LY-294002 from BabCO (Richmond, CA), antibodies against CD2 from Probes (Eugene, OR), antibodies against hemagglutinin (HA) from C-13 (Hamburg, Germany), antibodies against c-Src or phosphatidylinositol 3-kinase (PI 3-kinase) (Roche Molecular Biochemicals (Mannheim, Germany). The vectors expressing the HA-tagged FAK proteins (pHAFAK, pFAK-FLAG) was kindly provided by K. Yamada and H. Hirai (39).

Expression plasmids. The vectors expressing GFP and the GFP-FAK fusion proteins (pEGFP-FAK, pEGFP-FAT, and pEGFP-FRNK) were kindly provided by C. Damsky (25). The vectors pCD2, pCD2FAK, and pCD2FAK454 (lysine-phenylalanine) were kindly provided by A. Aruffo (10). The vectors expressing the HA-tagged FAK proteins pHAFAK, pHAFAK395 (aspartic acid-alanine), and pHAFAK397 (tyrosine-phenylalanine) were kindly provided by H. C. Chen (9). The vector expressing paxillin (pCEFL-HA-paxillin) was kindly provided by J. S. Gutkind (24). The vector expressing p130Cas (pSSRKeCas-FLAG) was kindly provided by K. Yamada and H. Hirai (39).

Transfections: BPAEC were transfected by a calcium phosphate procedure. Briefly, 24 h after the cells were plated, culture medium was replaced with fresh medium and cells were incubated for 1 h. DNA in 250 mM CaCl₂ was added to an equal volume of 2× HBS (270 mM NaCl, 10 mM KCl, 1.4 mM NaH₂PO₄, and 42 mM HEPES, pH 7.08) and incubated at room temperature for 30 min. In cases where a GFP cDNA was cotransfected, the ratio of effector DNA to GFP cDNA was 3:1 (wt/wt). The DNA-calcium phosphate mixture was then added to the cultures, and the cells were incubated for 5 h. The medium was removed, and the cultures were incubated with 15% glycerol-HBS for 2 min. The glycerol solution was removed and replaced with fresh medium, and the cells were cultured for an additional 24–48 h.

Apoptosis assay. Cells grown on coverslips in duplicate were washed three times in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min, and incubated for 10 min at room temperature with the DNA-specific stain Hoechst-33342 at 0.1 µg/ml. The coverslips were then mounted in an antifade solution (5% n-propyl gallate, 0.25% DABCO, and 0.0025% p-phenylenediamine in glycerol). The cells were then viewed by fluorescence microscopy (×200) using a fluorescence microscope (model E6400, Nikon). Successfully transfected cells, identified by GFP fluorescence or HA immunofluorescence, were classified as apoptotic or normal on the basis of nuclear morphology. Apoptotic cells had condensed, brightly staining chromatin, while normal cells had larger, faintly staining nuclei. Assays were conducted 16 h after treatment with Ado or Ado-Hc. For each coverslip, enough random fields were evaluated until the total number of GFP- or HA-positive cells analyzed was >100 at the end of a complete count of the last field. Results are expressed as percentage of transfected cells with apoptotic nuclei.

In some experiments, apoptosis was also assessed via TdT-mediated dUTP nick end labeling (TUNEL) staining as previously described (34). Briefly, cells grown on coverslips were washed in PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. After an additional wash in PBS, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C. After a wash in PBS, cells were washed with TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM cacodylic acid, 1 mM cobalt chloride, and 0.05% BSA) and then incubated in TdT buffer supplemented with 0.3 U/ml TdT and 1 µM biotinylated dUTP at 37°C for 1 h. Control samples were incubated in the absence of biotinylated dUTP. The cells were then washed and incubated with Texas red-conjugated streptavidin at room temperature for 1 h. The stained cells were washed, mounted, and viewed by fluorescence microscopy. Cells possessing brightly staining nuclei, as a result of TdT-mediated dUTP incorporation into fragmented DNA, were classified as apoptotic.

Immunoblot analysis. Immunoblot analysis was performed as previously described (22). Briefly, BPAEC were scraped and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 20 g/ml aprotinin. Protein was fractionated by SDS-PAGE, transferred to polyvinylidene fluoride, and analyzed by immunoblot essentially as described previously (22). Horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). Detection was achieved by chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacturer's instructions.

Immunofluorescence. Cells were cultured for immunofluorescence as previously described (22). Briefly, cells grown on coverslips were washed once with PBS, fixed for 10 min with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 7.2. After initial blocking, cells were incubated with primary antibodies in PBS/serum for 1 h at 37°C, washed, and incubated with fluoresceinated secondary antibody in PBS/serum. After final washing, cells were washed and mounted in antifade solution. Photographic images were obtained using a fluorescence microscope (model E6400, Nikon).

Statistical analysis. Values are means ± SD. Differences among the means were analyzed for significance by ANOVA plus Fisher's least significant difference test.
RESULTS

Overexpression of FAK, but not paxillin or p130Cas, blunts Ado-Hc-induced apoptosis. We previously demonstrated that Ado-Hc-induced endothelial cell apoptosis was accompanied by disruption of focal adhesion contacts and degradation of FAC proteins, including FAK, paxillin, and p130Cas (22). We therefore sought to determine whether ectopic expression of these proteins would blunt Ado-Hc-induced apoptosis. We employed an experimental approach of combining transient transfection of cDNA vectors expressing the proteins of interest with fluorescence analysis of transfected cells for apoptosis. We transfected exponentially growing BPAEC with expression vectors for FAK, paxillin, or p130Cas. As controls, the vectors lacking cDNA inserts were transfected. To confirm successful transfection, cells were analyzed by immunoblot and/or immunofluorescence. Figure 1 demonstrates that BPAEC transfected with a GFP-FAK construct had a punctate pattern of fluorescence, consistent with localization of GFP-FAK in FAC (Fig. 1, A and B). Immunoblotting with antibody to GFP revealed expression of GFP-FAK with the expected molecular mass (Fig. 1C). Similar analyses were performed for all constructs used in these studies, and confirmation of expression and localization to FAC was obtained for all constructs (data not shown).

At 24 h after transfection, cells were treated with Ado or Ado-Hc. After an additional 16 h of culture, cells were fixed and stained, and apoptosis was assessed by analysis of morphology of Hoechst-stained nuclei as described in MATERIALS AND METHODS. Figure 2 shows fluorescence analysis of a typical GFP-FAK transfection experiment. The GFP-FAK-expressing cell, identified by fluorescence, did not display condensed nuclear chromatin typical of apoptosis, in contrast to neighboring, non-GFP-fluorescent cells.

Cells transfected with paxillin or p130Cas cDNAs underwent apoptosis to an extent similar to vector-transfected cells (Fig. 3, A and B). However, overexpression of a wild-type, GFP-conjugated FAK blunted Ado- and Ado-Hc-induced apoptosis (Fig. 3C). GFP-FAK-expressing cells that were not apoptotic showed a punctate pattern of GFP fluorescence, consistent with maintenance of focal adhesions (Fig. 2). Thus overexpression of FAK, but not paxillin or p130Cas, blunts Ado-Hc-induced apoptosis.

To confirm that the inability of paxillin or p130Cas to protect from Ado-Hc-induced apoptosis was not specific to the particular method used to assess apoptosis, we repeated these experiments employing TUNEL staining. Cells were transfected with control vectors or vectors expressing paxillin or p130Cas. After 24 h, cultures were treated with Ado-Hc for 16 h, and apoptosis was assessed by fluorescence microscopy. The GFP-FAK-expressing cell, identified by fluorescence, did not display condensed nuclear chromatin typical of apoptosis, in contrast to neighboring, non-GFP-fluorescent cells.

Fig. 1. Expression of transiently transfected green fluorescent protein (GFP)-focal adhesion kinase (FAK) constructs in endothelial cells. Bovine pulmonary artery endothelial cells (BPAEC) grown on coverslips were transfected with vectors expressing GFP (A) or GFP-FAK (B). After 24 h, cells were fixed and viewed by fluorescence microscopy (×1,000) using a fluorescence microscope (model E6400, Nikon). Images were recorded with a digital camera. C: immunoblot analysis of cell lysates obtained from BPAEC transfected with vectors expressing GFP (lane 1) or GFP-FAK (lane 2). After an additional 16 h of culture, cell lysates were resolved by SDS-PAGE and immunoblotted for GFP.

Fig. 2. Analysis of apoptotic nuclei of transiently transfected endothelial cells. Fluorescence micrograph (×200) of a single field of cells viewed with filters appropriate for viewing GFP (A, 488-nm excitation and 509-nm emission) or Hoechst-stained DNA (B, 350-nm excitation and 460-nm emission), as indicated. Arrows, GFP-positive cell (A) with normal nuclear morphology (B). Other cells in B exhibit condensed, brightly staining nuclei characteristic of apoptosis. Cells were transfected with GFP-FAK.
assessed using TUNEL staining. Cells transfected with the pCEFL-HA control vector underwent apoptosis in response to buffer or Ado-Hc at a rate of 5.9 ± 3.1 or 46.9 ± 5.9%, respectively (n = 2). Cells transfected with pCEFL-HA-paxillin and treated with buffer or Ado-Hc underwent apoptosis at a rate of 5.4 ± 6.2 or 46.3 ± 5.9%, respectively (n = 2). Similar results were observed with p130Cas-transfected cells. Cells transfected with the pcDNA3 vector (control for p130Cas) underwent apoptosis in response to buffer or Ado-Hc at a rate of 5.7 ± 1.6 or 48.7 ± 14.4%, respectively (n = 2). Cells transfected with the p130Cas expression vector underwent apoptosis in response to buffer or Ado-Hc at a rate of 5.7 ± 1.0 or 44.0 ± 2.5%, respectively (n = 2). Thus, by two separate methods, we have demonstrated that neither paxillin nor p130Cas overexpression protects against Ado-Hc-induced endothelial cell apoptosis.

Central catalytic domain is required for protection from apoptosis. To determine which protein domains of FAK were necessary for protection, we compared the ability of various mutants to blunt apoptosis. FAT is a construct expressing only the focal adhesion-targeting domain; FRNK contains all the FAT sequences plus an additional 150 amino acids NH₂-terminus to FAT (Fig. 4) (25). Both of these deletion mutants lack the central catalytic domain but bind to integrins (8). Thus these mutants lack tyrosine kinase activity (25). We also transfected a full-length FAK cDNA fused to CD2 sequences or a mutant in lysine-454, which abrogates catalytic activity (10). The CD2 moiety targets the protein to membranes, yielding a constitutively active wild-type FAK. To identify successfully transfected cells for these constructs, which were not GFP fusions, we cotransfected a GFP expression vector. At 24 h after transfection, cells were treated with Ado or Ado-Hc, and after 16 h cells were assessed for apoptosis. Neither FAT nor FRNK elicited protection from Ado- or Ado-Hc-induced apoptosis (Fig. 5A). Overexpression of FAT induced some apoptosis in the absence of treatment, in agreement with other reports (25, 26). In addition, FAT increased Ado-induced apoptosis significantly compared with Ado-treated, GFP-expressing cells alone. FRNK did not induce apoptosis in the absence of treatment, in contrast to the effects seen with FAT. However, FRNK significantly increased the amount of apoptosis in response to Ado treatment. FAK with mutation at K454 did not protect, while CD2-FAK did blunt Ado- and Ado-Hc-induced apoptosis (Fig. 5B). These data support the notion that the catalytic activity of FAK is necessary for protection from Ado- and Ado-Hc-induced apoptosis.

FAK mutants incapable of signaling through PI 3-kinase are unable to prevent cell death. FAK undergoes autophosphorylation on tyrosine-397 and serves as a docking site for the SH2 domains of PI 3-kinase and Src (8). Survival signaling of FAK can proceed through either of these pathways depending on the cellular
FAK survival signals require phosphatidylinositol 3-kinase (PI 3-kinase) signaling. Consistent with results obtained using Hoechst staining, HA-FAK-transfected cells exhibited significant protection from Ado-Hc-induced apoptosis; however, neither the HA-FAK397 nor HA-FAK395 mutants were able to protect (Fig. 6B). Thus results obtained using TUNEL staining as an apoptosis assay further suggest that FAK-mediated cell survival requires signaling through PI 3-kinase.

**PI 3-kinase inhibition blocks the ability of FAK to protect.** We next sought to determine whether the ability of FAK to mediate protection required PI 3-kinase activity. We repeated our CD2-FAK rescue experiments in the presence of 25 μM LY-294002, an inhibitor of PI 3-kinase. In these experiments, we lowered the doses of Ado-Hc to 500 μM Ado or 50 μM Ado-Hc to better assess the effects of LY-294002. Ado and Ado-Hc enhanced apoptosis in cultures transfected with CD2 alone (Fig. 7). As in Fig. 5B, overexpression of CD2-FAK blunted Ado- and Ado-Hc-induced apoptosis. However, coincubation with LY-294002 increased apoptosis and blocked the ability of wild-type FAK to protect from apoptosis induced by Ado or Ado-Hc. These results suggest that PI 3-kinase activity is necessary to mediate protection against apoptosis.

We transfected cells with wild-type FAK, FAKY397F, or FAKD395A. As expected, overexpression of HA-FAK protected cells from apoptosis (Fig. 6A). The Y397F mutant failed to protect, suggesting that Src or PI 3-kinase binding may be necessary for protection. However, the D395A mutant, which retains Src binding and signaling, also failed to protect, suggesting that expression of a mutant capable of signaling through c-Src could not rescue endothelial cells from Ado-Hc-induced apoptosis. These results suggest that FAK-mediated protection against Ado-Hc-induced apoptosis may require signaling through PI 3-kinase.

To confirm these results using a different apoptosis assay, we repeated these experiments employing TUNEL staining. Consistent with results obtained using Hoechst staining, HA-FAK-transfected cells exhibited significant protection from Ado-Hc-induced apoptosis; however, neither the HA-FAK397 nor HA-FAK395 mutants were able to protect (Fig. 6B). Thus results obtained using TUNEL staining as an apoptosis assay further suggest that FAK-mediated cell survival requires signaling through PI 3-kinase.

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DISCUSSION

The data presented here indicate that FAK overexpression can protect endothelial cells from apoptosis in response to Ado and Ado-Hc. This result was observed with three distinct FAK constructs. In contrast, paxillin or p130Cas overexpression was ineffective. The inability of FAT, FRNK (both of which lack the catalytic domain), or K454 mutants (which via point mutation lacks catalytic activity) to protect against apoptosis suggests that a functional catalytic domain for FAK is required for this protection. The inability of Y397F mutants (a point mutation in the major autophosphorylation site) to protect suggests that tyrosine-397 phosphorylation is crucial for protection, an observation that is consistent with our previous observations that Ado-Hc-induced apoptosis is protein tyrosine phosphatase (PTPase) dependent (22). The inability of the Y397F (lacking Src and PI 3-kinase binding) or D395A (lacking PI 3-kinase binding but maintaining Src binding) mutants to protect, along with the ability of a PI 3-kinase inhibitor to potentiate Ado-Hc apoptosis and 2) prevent FAK rescue, suggests that PI 3-kinase binding and activity are required for protection.

Ado-Hc-induced endothelial apoptosis is accompanied by FAC disruption and caspase-dependent proteolysis of FAK, paxillin, and p130Cas (22). Normal cultured adherent cells receive survival signals from growth factors and ECM (5). ECM-initiated signals are mediated through integrins and associated kinases such as FAK (8). Our data suggest that Ado-Hc-mediated degradation of FAK prevents normal survival signaling from proceeding through FAK and PI 3-kinase and extends our knowledge with respect to the mechanisms of Ado-Hc-induced apoptosis.

FAK has previously been demonstrated to provide survival signals in canine kidney epithelial cells (9, 17), HL-60 cells (37), and fibroblasts (25). Apoptosis was induced by microinjection of Madin-Darby canine kidney cells with anti-FAK antibodies or peptides corresponding to a region of the integrin molecule thought to be required for FAK interaction (17). A membrane-tethered, and thereby constitutively activated, FAK rescues COS cells from apoptosis induced by loss of cell surface contact (10), and wild-type FAK overexpression protects serum-deprived primary fibroblasts from apoptosis (25). The catalytic activity of FAK enzyme and tyrosine-397 are necessary for FAK-mediated protection of fibroblasts from apoptosis, a finding consistent with our data (10, 25). However, the signaling mechanism by which FAK protects depends on cell type and culture conditions. Ilic and co-workers (26) demonstrated that binding of the SH3 domain of p130Cas to proline rich-1 region of FAK is required for rat synovial fibroblast survival when cultured on fibronectin (in the absence of serum). This signaling activates c-Jun NH2-terminal kinase via a Ras-Rac-Pak1-MKK4 pathway. Under these conditions, the PI 3-kinase pathway is not activated. However, they observed that if these cells are deprived of attachment in the presence of serum, signaling proceeds via a PI 3-kinase-Akt pathway. Thus it appears that extracellular survival signals through FAK are dependent on cell type and soluble factors.

The reason for protection from apoptosis by FAK overexpression is not clear. FAK can be cleaved in vitro by caspases, and Ado-Hc-induced FAK degradation is caspase dependent (22). It is possible that the presence of excess FAK acts as a competitive inhibitor of caspases and stabilizes focal adhesions by preventing caspase-dependent proteolysis, thus preserving normal PI 3-kinase signaling. Alternatively, FAK overexpression may act as a competitor of some other enzyme, such as a PTPase, thus increasing FAK phosphorylation and maintaining PI 3-kinase signaling. Our previous data implicate a PTPase in Ado-Hc-induced apoptosis and would be consistent with this alternative (21, 22).

The inability of paxillin or p130Cas overexpression to protect suggests that, under these conditions, these FAK-associated proteins do not mediate survival signals, although evidence suggests that they are involved in signaling. Paxillin was first identified as a cytoskeletal protein with increased tyrosine phosphorylation in Src-transformed fibroblasts (4, 19). Paxillin becomes phosphorylated on tyrosine residues in response to a variety of stimuli, including bombesin, platelet-derived growth factor, and cell adhesion (32, 42, 44). It is believed to act as a scaffolding protein in FAC by mediating interactions with other signaling or cytoskeletal proteins (41), suggesting that paxillin may be involved in diverse signaling pathways. Indeed, phosphorylation of paxillin on tyrosines-31 and -118 regulates rat bladder carcinoma cell migration (30).

Recent data also implicate p130Cas in mediating intracellular signaling. As a member of a recently identified family of proteins that serve in mediating cytoskeletal signaling pathways, p130Cas undergoes increased tyrosine phosphorylation in Src-transformed fibroblasts and serves as an adapter protein with one SH3 domain and multiple tyrosines in SH2 consensus motifs to bind many different signaling proteins (24, 25). In our assay, however, wild-type p130Cas is unable to rescue BPAEC from Ado-Hc-induced apoptosis, although it is effective for FAK-mediated protection from Ado-Hc-induced endothelial cell apoptosis (25). This may indicate that the SH3 domain of p130Cas is not a sufficient binding site for FAK in this model system.
sites (29, 35). p130Cas binds to and is phosphorylated by FAK. p130Cas has been implicated in G protein-coupled receptor signaling, in growth factor receptor stimulation, and in antigen receptor stimulation (29). Ilic and co-workers (26) observed that binding of the SH3 domain of p130Cas to proline-rich-1 region of FAK is required for fibroblast survival on fibronectin in the absence of serum.

Although paxillin and p130Cas can participate in intracellular signaling, we observe no evidence that they participate in survival signaling required in Ado-Hc-treated endothelial cells. This may be due to the fact that they are structural proteins, in contrast to FAK, which has enzymatic activity. Alternatively, degradation of endogenous FAK may prevent the association of overexpressed paxillin and p130Cas into complexes necessary for providing survival signals.

The downstream effector of FAK survival signals in Ado-Hc-treated cells is PI 3-kinase, an enzyme that is required for multiple cellular processes, including cell proliferation, differentiation, and apoptosis (40). PI 3-kinase comprises a family of agonist-stimulated lipid-signaling enzymes that initiate signaling cascades by generating three distinct membrane phospholipids, the phosphoinositides phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. Many enzymes, including protein kinases, phospholipases, and G proteins are effector molecules of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, and their activities are affected by lipid-protein interaction (7). The regulatory unit, p85, of PI 3-kinase contains an NH2-terminal SH3 domain, a breakpoint cluster homology (BH) domain, and two SH2 domains. These domains allow p85 to simultaneously interact with multiple intracellular signaling molecules. Thus PI 3-kinase can recruit a variety of signaling molecules. There are several phosphoinositide-binding proteins that are potential downstream effectors of PI 3-kinase. One potential mechanism is activation of Akt (also known as protein kinase B), a serine/threonine kinase that is recruited to the plasma membrane (7). At the plasma membrane, the PI 3-kinase effector phosphatidylinositol-dependent kinase-1 (PDK1) phosphorylates Akt at threonine-308 and serine-473 (1, 16). Activated Akt can provide survival signals through various downstream pathways, including nuclear factor-κB activation, phosphorylation and inactivation of the proapoptotic Bcl-2 family member BAD, forkhead transcription factors (FKHR, FKHRL1, and AFX), glycogen synthase kinase-3β (GSK-3β), CREB, and caspase 9 (40). Thus there are multiple potential pathways regulating FAK and PI 3-kinase to cell survival.

In summary, overexpression of FAK protects endothelial cells from apoptosis induced by Ado-Hc, and this protection is dependent on PI 3-kinase activity and binding. Thus FAK signaling through PI 3-kinase may be important in regulation of endothelial cell survival. Better understanding of factors regulating endothelial cell survival may result in improved treatments of acute lung injury.

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