Extracellular matrix regulates glomerular epithelial cell survival and proliferation

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Bijian, Krikor, Tomoko Takano, Joan Papillon, Abdelkrim Khadir, and Andrey V. Cybulsky. Extracellular matrix regulates glomerular epithelial cell survival and proliferation. Am J Physiol Renal Physiol 286: F255–F266, 2004.—Glomerular epithelial cell (GEC) injury and apoptosis may contribute to sclerosis in glomerulonephritis. The present study addresses signals that regulate survival of GEC in culture and in the acute puromycin aminonucleoside nephrosis (PAN) model of GEC injury in vivo. Compared with GEC on plastic substratum, adhesion to collagen increased activation of focal adhesion kinase (FAK), c-Src, and ERK and facilitated survival (prevented apoptosis). GEC on plastic exhibited increased caspase-8 and -9 activities, increased expression of the proapoptotic protein, Bax, and decreased the antiapoptotic protein, Bcl-xL, compared with collagen. Stable expression of constitutively active mutants of FAK (CD2-FAK) or MEK (R4F-MEK) activated the ERK pathway and supplanted the requirement of collagen for survival. In contrast, expression of a Ras mutant that activates phosphatidylinositol 3-kinase but blocks ERK activation or pharmacological inhibition of the ERK pathway decreased survival on collagen. Glomeruli isolated from rats with PAN revealed increased β1-integrin expression, along with increased activation of FAK, c-Src, and ERK, compared with controls. EGF receptor activation was undetectable in PAN. Therefore, adhesion to collagen, resulting in activation of FAK and the Ras-ERK pathway, supports GEC survival. Analogous signals for GEC survival are activated in PAN.

apoptosis; glomerulonephritis; protein kinases; signal transduction

CELL HOMEOSTASIS REFLECTS a balance of proliferation, apoptosis, and differentiation (20, 21, 26). Apoptosis may be triggered by insufficient exposure to growth factors, disruption of the cytoskeleton, inadequate ECM-cell contact (also known as “anoikis”), and other factors (20). Mechanisms reported to regulate cell survival (prevention of apoptosis/anoikis) may involve adhesion receptors, such as integrins, and protein or lipid kinases, including focal adhesion kinase (FAK), ERK, phosphatidylinositol 3-kinase (PI3K)-Akt, or integrin-linked kinase pathways, as well as multiple downstream effectors, including Bcl-2 family proteins, caspases, nuclear factor-κB, and others (6, 20, 21, 26, 38, 41). Signals from ECM may directly affect cellular functions or may interact with signals from growth factors (41). FAK appears to play a major role in mediating signals emanating from ECM via β1-integrins. In cultured cells, FAK may be recruited to focal contacts directly or through cytoskeletal proteins, talin and paxillin, where FAK interacts with the cytoplasmic tail of the β1-integrin subunit (6). Activation of FAK leads to autophosphorylation at Tyr397, creating a binding site for c-Src. On binding, c-Src phosphorylates Tyr925 of FAK, allowing Grb2, the adaptor protein, to bind to the motif surrounding Tyr925. Grb2, which exists as a complex with Sos, may link integrin signals to the Ras-ERK pathway (6, 24, 39, 40). Phosphorylation/activation of FAK may affect various cellular responses (6). For example, activation of FAK was able to prevent apoptosis, although the mechanism for this effect remains unclear. One possibility involves the binding of the Src homology 2 domain of PI3K to Tyr539 of FAK, thus promoting survival through the PI3K-Akt pathway, or by the inhibition of proapoptotic proteins, such as p53. The role of ERK in mediating downstream signals from FAK has not been well defined.

Glomerular visceral epithelial cells (GEC) or podocytes are intrinsic components of the kidney glomerulus and play a key role in the maintenance of glomerular permselectivity (19, 27, 35). Under normal conditions, where GEC are in contact with ECM and are exposed to trivial concentrations of growth factors, there appears to be little turnover of GEC. Certain forms of glomerulonephritis feature GEC injury, which may lead to apoptosis or proliferation, and, in association with ECM expansion, to glomerulosclerosis and/or impaired glomerular function or permselectivity. Cross talk between ECM receptors and growth factor receptors may modulate GEC proliferation and/or survival. We have previously determined that EGF, basic fibroblast growth factor, and hepatocyte growth factor stimulate proliferation of cultured GEC that are adherent to collagen, but not plastic substratum (12, 13). Moreover, in GEC on collagen, EGF was able to induce sustained activation of the EGF receptor (EGF-R) and ERK, in association with reduced EGF-R dephosphorylation by phosphatases. On plastic, EGF-R activation was weak or transient, as dephosphorylation appeared to be rapid (12, 13).

In vivo, GEC injury occurs in human focal segmental glomerulosclerosis (FSGS), in the context of ECM expansion (4, 5, 36, 46). Familial forms of FSGS, associated with mutations in GEC structural proteins, further support the view that FSGS is a disease of the GEC (19, 27, 35). FSGS may feature apoptosis as well as proliferation; the latter is observed in the collapsing variant of FSGS, where the proliferating cells appear to be visceral GEC with dysregulated phenotype and loss of the p27 cyclin-dependent kinase inhibitor. Experimental rat models, including acute and chronic puromycin aminonucleoside nephrosis (PAN), have provided a better understanding of the pathophysiology of GEC injury in FSGS (22). These models generally feature GEC injury with heavy proteinuria, sclerosis, and some GEC detachment, expansion of ECM components, as well as collapse of glomerular capillaries in chronic PAN (18, 32, 42). DNA synthesis in GEC occurred early and late in the course of PAN, with increasing GEC...
apoptosis, and loss of GEC as the lesion became more chronic (42). Actually, it has been proposed that GEC apoptosis may lead to “podocytopenia” and consequently sclerosis in FSGS (19, 27, 28, 35). Alterations in the expression of ECM structural proteins and filtration slit-diaphragm components have also been reported (19, 27, 32). Together, these changes suggest that in PAN, there may be altered signaling from the ECM to GEC, but precisely how such signals may regulate GEC apoptosis or proliferation requires further study.

The aim of the present study was to address the signals that regulate survival/proliferation of GEC in culture and to determine whether analogous signals are activated in the PAN model of GEC injury in vivo. We demonstrate that adhesion of GEC to collagen enhances activation of FAK, c-Src, and ERK and prevents apoptosis via the ERK pathway. Stable expression of constitutively active mutants of FAK or MEK supplanted the requirement of ECM for promoting survival, and in the presence of growth factors, proliferation. Analogous signals for GEC survival are activated in glomeruli of rats with PAN.

MATERIALS AND METHODS

Materials. Tissue culture reagents, the Transfinitty CaPO4, transfection system, G418 (geneticin), RNase A, and proteinase K were obtained from Invitrogen (Burlington, ON). Pepsin-solubilized bovine dermal collagen I (Vitrogen) was from Cohesion (Palo Alto, CA). EGF, thrombin, puromycin aminonucleoside, Engelbreth-Holm-Swarm sarcoma laminin, LY-294002, and rabbit anti-actin antibody were obtained from Sigma (St. Louis, MO). Rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) and rabbit anti-ERK1/2 antibodies were purchased from New England Biolabs (Mississauga, ON). NuSerum, collagen IV, and mouse monoclonal antibodies to phosphotyrosine (PY20), Ras, Bax (6A7), and Bcl-Xl were obtained from BD Biosciences (Mississauga, ON). GST-Grb2 (1-217) fusion protein, rabbit anti-Grb2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-MEK1 was purchased from Stressgen Biotechnologies (Victoria, BC). Protein A-agarose and mouse anti-Src monoclonal antibody were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-phospho-Src (Tyr419) was purchased from Biosource International (Camarillo, CA). Bisbenzimide H-33242 fluorochrome, propidium iodide, caspase-8 substrate (granzyme B substrate I), caspase-9 substrate II, LEHD-CHO, PP2, and PD-98059 were obtained from Calbiochem (La Jolla, CA). Rabbit anti-EGF-R antibody (RK2) was described previously (14). Anti-β1-integrin rabbit antiserum was kindly provided by Dr. Michael DiPersio (Albany Medical College, Albany, NY) (34). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, ON). Male Sprague-Dawley rats (150 g) were purchased from Charles River Canada (St. Constant, PQ). An expression plasmid containing G12V/Q43E/D54N/E63K Ras mutant (J25 Ras) cDNA was kindly provided by Dr. Anne Vojtek (University of Michigan, Ann Arbor, MI) (48). Plasmid CDM8 containing constitutively active FAK (CD2-FAK) cDNA was kindly provided by Dr. Alejandro Aruffo (Bristol Myers Squibb, Princeton, NJ) (7). R4F-MEK cDNA was provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO) (33).

ECM and GEC culture. Type I collagen gel matrices were prepared as described previously (9). Collagen IV solution was applied to culture wells at 0.02 mg/cm² and allowed to air-dry at 22°C. Collagen I was used in all experiments, except where indicated. Primary cultures of rat GEC were established from explants of rat glomeruli and have been used extensively previously (8, 9, 12, 13). Unlike some other GEC culture lines, the cells used in the present study do not contain the SV40 large T antigen and thus in our view are better suited for studies of cell survival. Experiments were done with cells between passages 25 and 70. Under standard conditions, GEC were cultured on collagen I matrices in K1 medium, which consisted of DMEM/Ham F-10 (1:1) containing 5% NuSerum and hormone supplements (9, 12). GEC were maintained in culture by passaging onto collagen gels; for experiments, GEC were replated onto collagen, laminin, or plastic substratrum in serum-poor (DMEM-0.5% fetal calf serum) or K1 media.

GEC transfection. GEC on collagen were cotransfected with the expression vector of interest (2 μg of DNA/100-mm plate) and a plasmid containing resistance gene pRCRSV (molar ratio 12:5:1), using the CaPO4 technique as described previously (14). GEC were then cultured on collagen in K1 medium containing 0.5 mg/mL G418. GEC clones resistant to G418 were isolated and replated onto plastic substratrum. Clones that proliferated on plastic in K1 medium were selected, passaged, and assessed for the expression of the specific protein by immunoblotting. A soft agar assay was used to test for anchorage-independent proliferation (14).

Measurement of GEC proliferation. Cell number was determined by visual counting. Cells adherent to collagen gels (35-mm plates) were placed into single-cell suspension with collagenase and trypsin-EDTA. Cells on plastic substratum were placed into suspension by incubation with trypsin-EDTA. Suspended cells were then counted in a hemacytometer (12, 14).

Induction of PAN in rats. PAN was induced in male Sprague-Dawley rats (150 g) by a single intravenous injection of 80 mg/kg of puromycin aminonucleoside (22). Urine was collected on day 14. The rats were then killed, and glomeruli were isolated by differential sieving (15).

Immunoprecipitation and immunoblotting. Preparation of GEC and glomerular lysates was described previously (15). Briefly, after treatment, ~6 × 10⁶ GEC were lysed in immunoprecipitation buffer, containing (in mM) 125 NaCl, 20 Tris, 20 0.2 PMSF, 25 NaF, 2 Na3VO4, 5 Na4P2O7, and 1 EDTA, pH 7.4 (4°C), as well as 20 μM leupeptin, 20 μM pepstatin, and 1.0% Triton X-100. The mixture was centrifuged at 14,000 g for 10 min, and the supernatant was then used for immunoprecipitation or immunoblotting. Glomeruli were centrifuged and resuspended in immunoprecipitation buffer as above. After protein concentrations were adjusted, proteins were immunoprecipitated with primary antiserum under quantitative conditions, as described previously (15). Immune complexes were incubated with agarose-coupled protein A. Complexes were boiled in Laemmli sample buffer and subjected to SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 3% BSA/2% ovalbumin, and incubated first with primary antibody and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminesence technique (Amersham Pharmacia Biotech). Protein content was quantified by scanning densitometry, using NIH Image software (15). Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

DNA fragmentation. Adherent cells were collected in PBS and centrifuged for 5 min at 500 g (4°C). Cell pellets were resuspended in buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.2% Triton X-100, pH 7.4, and incubated for 30 min (4°C). The mixture was centrifuged at 14,000 g for 10 min. The supernatant was collected and incubated with 100 μg/mL of RNase A and 100 μg/mL of proteinase K for 1 h (37°C). Ice-cold 5 M NaCl and isopropanol were added to the mixture to precipitate the DNA overnight (~20°C). The pellet was then washed in ice-cold ethanol and dissolved in Tris-EDTA buffer. After DNA concentrations were adjusted, 3 μg of DNA were separated on a 1.2% agarose gel prestained with ethidium bromide.

Hoechst H-33342 staining. To quantitate apoptosis, adherent cells were stained with Hoechst H-33342 dye (1 μg/mL) for 10 min at 37°C without fixation (31). After being washed with PBS, cells were stained with propidium iodide (5 μg/mL) to visualize necrotic or “late-apoptotic” cells. Nuclei of apoptotic cells were condensed and stained
brightly with H-33342 dye, whereas propidium iodide was excluded. Cells were photographed using a Nikon Diaphot immunofluorescence microscope and Nikon Coolpix 995 digital camera, and the number of H-33342-positive/propidium iodide-negative cells were quantified by visual counting.

Caspase activity assay. Intracellular caspase-8 and -9 activities were measured using a colorimetric assay with p-nitroaniline (pNA)-labeled substrates, according to the manufacturer’s instructions. Adherent cells were lysed in buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, and 10% glycerol, pH 7.4 (4°C), with a 2 mM substrate, i.e., Ac-Leu-Glu-His-Asp-pNA (caspase-9), or Ac- Ile-Glu-Thr-Asp-pNA (caspase-8). The absorbance of the reaction was read at 405 nm every 5 min for 2 h. After subtraction of background activity, the slope of absorbance vs. time is proportional to caspase activity.

Immunofluorescence microscopy. Cryostat kidney sections (4 μm) were fixed with ether-ethanol (1:1, 10 min), followed by ethanol (20 min) at 4°C. Sections were incubated with rabbit anti-phospho-ERK IgG (2.5 μg/ml) overnight at 4°C. Nonimmune rabbit IgG was used in control incubations. After being washed, sections were incubated with fluorescein-conjugated goat anti-rabbit IgG for 1 h at 22°C. The immunofluorescence signals were evaluated using a Nikon Diaphot immunofluorescence microscope with visual output connected to a Nikon Coolpix 995 digital camera.

Statistics. Data are presented as means ± SE. The t-statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t-statistic and adjusting the critical value according to the Bonferroni method.

RESULTS

Collagen facilitates GEC survival. In keeping with previous results (12, 13), addition of growth factors (K1 medium) or EGF to cultured GEC adherent to collagen resulted in proliferation (as reflected by a progressive increase in cell number) (Fig. 1). Cell number remained constant in GEC on collagen in the presence K1 medium or EGF. In the absence of growth factors, cell number was sustained on collagen. Cell number decreased on plastic, independently of the culture media. *P < 0.001, collagen K1 vs. plastic K1. **P < 0.001, collagen SP vs. plastic SP. ***P < 0.001 collagen EGF vs. plastic EGF (8 experiments).

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To determine whether the decline in GEC number in the absence of collagen was due to apoptosis, we analyzed DNA isolated from parental GEC plated onto collagen or plastic in the presence or absence of growth factors. DNA fragmentation (“laddering”), a feature consistent with apoptosis, was evident in parental GEC adherent to plastic after 72 h of plating, but not in GEC plated onto collagen (Fig. 2A). DNA laddering occurred whether growth factors were or were not present in the medium. In addition, we quantified the amount of apoptosis by staining GEC with H-33342 dye and propidium iodide. In this assay, nuclei of apoptotic cells (which show chromatin condensation and fragmentation) stain brightly with H-33342, these cells generally do not stain with propidium iodide, because apoptotic cells usually possess intact plasma membranes. However, in cell culture, propidium iodide-positive cells are generally late-apoptotic, as apoptotic cells are not phagocytosed and may proceed to necrosis. H-33342-labeled GEC were evident almost exclusively in monolayers of cells adherent to plastic (Fig. 2, B and C), consistent with the DNA laddering assay. The presence of growth factors did not affect the amount of apoptosis in GEC on plastic (Fig. 2C), even though growth factors slightly attenuated the decline in cell number (Fig. 1). Possibly, the H-33342 assay was not sufficiently sensitive to detect minor differences. Substitution of collagen with laminin was unable to promote GEC survival, shown by increased numbers of H-33342-positive cells in GEC plated onto laminin, compared with GEC on collagen (Fig. 2D). These results highlight a key role for collagen in promoting survival.

Effect of collagen on caspase activity and expression of Bax and Bcl-XL. The morphological changes in an apoptotic cell are induced by the activation of caspases (43) and may be modulated by pro- or antiapoptotic actions of the Bcl-2 family of proteins. Caspase-9 activity (linked to release of caspase-9-activating proteins from mitochondria into the cytosol) was increased in GEC adherent to plastic, compared with collagen (Fig. 3A). Interestingly, caspase-8 activity (typically linked to “death domain” receptors) was also increased in GEC adherent to plastic (Fig. 3A). Pretreatment of GEC with the caspase-9 inhibitor LEHD-CHO (30) blocked both plastic-stimulated caspase-9 and -8 activities (caspase-9: collagen 0.11 ± 0.02, plastic 0.76 ± 0.14, plastic + inhibitor 0.33 ± 0.05; caspase-8: collagen 0.11 ± 0.03, plastic 0.77 ± 0.15, plastic + inhibitor 0.34 ± 0.02; n = 4, P < 0.0025, plastic + inhibitor vs. plastic for both caspases; activity is presented in arbitrary units). Finally, analysis of the expression of two proteins of the Bcl-2 family revealed a significant increase in the proapoptotic protein Bax and a significant decrease in the antiapoptotic protein Bcl-XL in GEC plated onto plastic substratum compared with GEC on collagen (Fig. 3B).

Adhesion to collagen induces activation of FAK and ERK. The next series of studies addressed collagen-induced signals that may mediate survival. First, we examined activation of FAK. FAK protein expression and tyrosine phosphorylation (which reflects FAK activation) were increased on collagen compared with plastic substratum (Fig. 4A). The change in FAK phosphorylation exceeded the increase in expression (Fig. 4A, right). FAK activation was due to a direct effect of
collagen, as EGF or K1 medium was unable to activate FAK (data not shown).

The nonreceptor protein tyrosine kinase c-Src is reported to regulate FAK activity (6). In parallel with FAK, c-Src Tyr 418 phosphorylation (reflecting activation of Src) was increased significantly in GEC adherent to collagen compared with plastic substratum (Fig. 4B). Treatment of parental GEC on collagen with PP2, a selective inhibitor of the Src family of protein tyrosine kinases (25), significantly inhibited FAK phosphorylation, compared with untreated GEC (Fig. 4C).

In addition to FAK, adhesion of GEC to collagen stimulated an approximate twofold increase in ERK1 and ERK2 Thr 202/Tyr 204 phosphorylation (Fig. 4D), which reflects ERK activation (12, 14). Collagen did not significantly alter ERK protein expression (Fig. 4D). The phosphorylation of ERK was a direct effect of collagen and was not dependent on the presence of growth factors in the culture medium. The reduction in FAK phosphorylation by the PP2 Src inhibitor (Fig. 4C) was paralleled by a reduction in ERK phosphorylation (Fig. 4E; 40% inhibition by densitometry). These results suggest that FAK is linked to and may signal via the ERK pathway.

Expression of constitutively active FAK and MEK. To determine the downstream targets of FAK, and whether FAK activation was functionally important, GEC on collagen were stably cotransfected with a neomycin resistance gene plus constitutively active FAK cDNA (CD2-FAK). Of 68 G418-resistant clones that proliferated on collagen in K1 medium, 34 were also able to proliferate on plastic, and all of these clones expressed CD2-FAK at various levels (results from representative clones are shown in Fig. 5A). Thus expression of CD2-FAK supplanted the requirement for collagen shown in Fig. 1.

In the next series of experiments, we tested whether ERK is a potential target of endogenous FAK and CD2-FAK, i.e., whether activated FAK or CD2-FAK could bind Grb2, an adapter protein that would link FAK to the Ras-ERK pathway. FAK was immunoprecipitated from parental GEC on collagen or plastic, or from CD2-FAK-transfected GEC, and the immunoprecipitates were blotted with either anti-phosphotyrosine antibody or GST-Grb2, followed by anti-Grb2 antibody. The results demonstrate that CD2-FAK, as well as FAK in GEC on collagen, were intensely tyrosine phosphorylated (activated) and that only the intensely phosphorylated CD2-FAK and FAK bound Grb2 (Fig. 5B).

To examine the functional role of the Ras-ERK pathway, GEC on collagen were stably cotransfected with a neomycin resistance gene plus constitutively active MEK (R4F-MEK; N3/S218E/S222D). Among multiple G418-resistant clones that proliferated on collagen in K1 medium, 17 clones were also able to proliferate on plastic substratum in a growth factor-dependent manner, and all of these clones expressed R4F-MEK. Three representative clones are shown in Fig. 5C (it should be noted that endogenous MEK was not readily detectable by immunoblotting). In the absence of exogenous EGF (or other mitogens), ERK phosphorylation in the three R4F-MEK-expressing GEC clones was at least fivefold greater compared with parental GEC on collagen (4 experiments) (Fig. 5D). By analogy to the stimulatory effect of collagen in parental GEC, as well as the effect of R4F-MEK expression, ERK phosphor-
ylation was readily detectable in the CD2-FAK-expressing cells in the absence of exogenous EGF (Fig. 5E, first 4 lanes).

FAK and the ERK pathway mediate GEC survival. Adhesion to collagen induced ERK activation, whereas expression of constitutively active FAK and MEK supplanted the collagen effect. In parallel, R4F-MEK- or CD2-FAK-expressing GEC plated on plastic showed levels of apoptosis (determined by H-33342 staining) similar to parental GEC on collagen; i.e., levels of apoptosis in these clones were significantly lower compared with parental GEC on plastic (Fig. 6, untreated). In addition, DNA fragmentation was not observed in the GEC that express CD2-FAK (Fig. 2A). To verify that the prosurvival signaling of collagen was actually mediated through the ERK pathway, GEC clones were treated with the MEK inhibitor (data not shown). PI3K inhibition significantly reduced EGF-dependent ERK activation (on collagen), which supplants collagen) (12) may potentially activate other effectors (e.g., PI3K) is sufficient to supplant collagen. J25 Ras expression significantly reduced EGF-dependent ERK activation (on collagen), which proceeds via EGFR-Ras-Ras (Fig. 8B), but not activation by thrombin, which stimulates ERK via a G protein receptor, independently of Ras (Fig. 8, C and D). In the absence of EGF or thrombin, ERK phosphorylation was lower in J25 Ras-expressing GEC on collagen compared with parental cells (Fig. 8, B and C). These results imply that J25 Ras functioned as a dominant inhibitor of the Ras-ERK pathway, blocking both EGF- and collagen-stimulated ERK phosphorylation.

Growth factor signals are regulated by collagen. The experiments described above focus on signals induced by collagen, which regulate GEC survival. Previously, we demonstrated that in the presence of growth factors, including EGF, GEC proliferate when adherent to collagen I or IV matrices, but not plastic substratum, and that stable transfection of constitutively active Ras (V12Ras) supplanted the requirement for collagen, but not for growth factors (12-14). Similar to previous results (13), EGF further stimulated ERK activation in GEC on collagen I but was not able to activate ERK significantly on plastic (Fig. 7, A and B). EGF also stimulated ERK phosphorylation on collagen IV (a more physiological substratum for GEC) (Fig. 7A). Moreover, in the CD2-FAK-expressing GEC and in the R4F-MEK-expressing clones, ERK phosphorylation was further stimulated by EGF (Figs. 5E and 7B), similar to ERK activation in collagen-adherent parental GEC, but unlike parental GEC on plastic (Fig. 7B). These results are similar to those observed earlier in GEC that were stably transfected with V12Ras, a constitutively active Ras mutant (14). Thus constitutively active Ras, FAK, or MEK provided sustained ERK activation and enabled EGF to further stimulate ERK activity in the absence of collagen.

Proliferation of R4F-MEK- and CD2-FAK-expressing clones occurred independently of collagen but was nonetheless dependent on adhesion to plastic (i.e., anchorage-dependent), and required addition of exogenous growth factors (Fig. 7, C and D). Thus R4F-MEK and CD2-FAK were able to supplant the requirement for collagen, but not growth factors, and the ability of EGF to induce proliferation correlates with its ability to induce ERK activation (Fig. 7B). R4F-MEK- and CD2-FAK-expressing GEC clones did not proliferate in soft agar (data not shown), indicating that these cells were not transformed.

ERK is the principal Ras effector that mediates GEC survival. In addition to the ERK pathway, collagen or V12Ras (which supplants collagen) (12) may potentially activate other downstream effectors. To determine whether the PI3K pathway may be activated by collagen, or alternatively, whether constitutive PI3K activation would supplant the signal from collagen, GEC on collagen were stably transfected with J25 Ras (Q43E/D54N/E63K Ras), a Ras mutant that constitutively activates PI3K but is unable to activate the ERK pathway (Fig. 8A). In the presence of growth factors, J25 Ras-expressing GEC were unable to proliferate or survive on plastic, suggesting that activation of non-ERK Ras effectors (e.g., PI3K) is insufficient to supplant collagen. J25 Ras expression significantly reduced EGF-dependent ERK activation (on collagen), which proceeds via EGFR-Ras-Ras (Fig. 8B), but not activation by thrombin, which stimulates ERK via a G protein receptor, independently of Ras (Fig. 8, C and D). In the absence of EGF or thrombin, ERK phosphorylation was lower in J25 Ras-expressing GEC on collagen compared with parental cells (Fig. 8, B and C). These results imply that J25 Ras functioned as a dominant inhibitor of the Ras-ERK pathway, blocking both EGF- and collagen-stimulated ERK phosphorylation.
Culture of J25 Ras-expressing GEC in growth factor-poor medium (on collagen) resulted in a marked decrease in cell number, whereas cell number remained constant in parental GEC under similar conditions (Fig. 8E). Together with the observed reduction in ERK phosphorylation in J25 Ras-expressing GEC on collagen (Fig. 8B, no EGF), these results indicate that J25 Ras most likely blocked a signal for GEC survival from the collagen matrix, involving the Ras-ERK pathway. This inhibitory action of J25 Ras was unrelated to growth factor-induced Ras-ERK activation, because growth factors were absent in these experiments (Fig. 8E). The above results demonstrate the importance of the ERK pathway in mediating the survival signals emanating from collagen. Possibly, PI3K may also contribute to GEC survival (Fig. 6, LY-294002), but this pathway is unable to mediate survival independently of the ERK pathway.

In the presence of growth factors (K1 medium), GEC that express J25 Ras proliferated on collagen, but at a reduced rate compared with parental cells. Parental GEC passed at a ratio of 1:10 reached 50% confluence at 4.3 ± 0.1 days (n = 11), whereas J25 Ras-expressing GEC passed at a ratio of 1:3 reached 50% confluence only at 7.7 ± 1.2 days (n = 6, P < 0.001). The result confirms that J25 Ras most likely functioned as a dominant inhibitor of endogenous Ras and blocked EGF-induced activation of the ERK pathway.

Signals for GEC survival are activated in PAN. The above studies characterized signaling by ECM and growth factors in cultured GEC, but it is important to demonstrate that analogous signaling pathways may be activated in GEC in vivo. To address this question, we employed the acute PAN model of GEC injury (22). First, we demonstrated that puromycin aminonucleoside markedly enhanced the apoptotic potential of cultured GEC on plastic (Fig. 9). On collagen, there was an upward trend in apoptosis in the presence of puromycin aminonucleoside, but adhesion to collagen largely protected GEC from puromycin aminonucleoside-induced apoptosis (Fig. 9). These results suggest that the collagen-induced survival signals characterized above (Figs. 4–6) can limit GEC injury. Treatment of GEC on collagen with puromycin aminonucleoside did not enhance the level of collagen-induced ERK phosphorylation significantly (puromycin aminonucleoside: 92% of untreated at 30 min, 115% at 2 h, and 99% at 24 h; n = 4). In vivo, 2 wk after administration of puromycin aminonucleoside, rats demonstrated marked proteinuria, a hallmark of GEC injury (control 8 ± 3, PAN 246 ± 29 mg protein/24 h). Expression of β1-integrin was increased almost twofold in glomeruli isolated from rats with PAN compared with controls (Fig. 10A), suggesting that in PAN, there may be amplification of those signals from ECM, which are mediated via β1-integrins. Protein expression and phosphorylation of FAK (Fig. 10B), c-Src (Fig. 10C), and ERK (Fig. 10D) were detectable in normal (control) glomeruli, and significant increases in activation and expression of these proteins were evident in glomeruli isolated from rats with PAN compared with controls. In contrast, EGF-R protein expression was not significantly different between control and PAN, and EGF-R tyrosine phosphorylation was not detected consistently in either group (Fig. 10E). Thus the protein kinases that are activated by collagen and support survival in cultured GEC are also activated in vivo.

By immunofluorescence microscopy, glomeruli of rats with PAN showed specific, although faint staining for phos-
with (H11002) or without (H11001) EGF (100 ng/ml) for 30 min. Equal amounts of total protein were loaded into each lane before immunoblotting with anti-phospho-ERK.

Staining of glomerular capillary loops was evident, particularly at the periphery of glomeruli, in keeping with ERK phosphorylation in all cell lines (Figs. 2A and 6), implying that FAK activation is antiapoptotic. As reported in other cells (40), collagen-induced FAK activation in GEC was, at least in part, dependent on the activation of c-Src (Fig. 4,B and C). Activated endogenous FAK in parental GEC on collagen and CD2-FAK were able to bind Grb2, but nonphosphorylated FAK in parental GEC on plastic did not (Fig. 5B). In addition, adhesion of GEC to collagen or expression of CD2-FAK increased ERK phosphorylation (Figs. 4D and 5E).

**DISCUSSION**

This study demonstrates that adhesion of GEC to collagen facilitates survival, principally via activation of FAK and a Ras-ERK pathway. After plating of GEC onto collagen in the absence of growth factors, cell number remained constant, whereas cell number declined on plastic substratum, and GEC demonstrated features of apoptosis (Figs. 1 and 2). It should be noted that the proapoptotic effect of plastic was not due to the inability of GEC to attach and spread on plastic. Surprisingly, adhesion of GEC to laminin did not facilitate survival (Fig. 2D), even though GEC express laminin-binding cell surface receptors (1, 10, 11). The major cell surface receptors for ECM proteins are integrins, and GEC in culture and in vivo express αβ1-integrin (1, 10, 11). Cultured GEC adhere to collagen I, collagen IV, laminin, and fibronectin and show greater adherence to the collagens, compared with the other substratum (1, 11). Anti-β1-integrin antibody significantly inhibited adhesion to ECM proteins (1, 11). Thus the antiapoptotic effect of collagen may be related to the greater efficiency of GEC adhesion to this ECM protein.

FAK, a key intermediate protein kinase that transmits some of the signals from ECM that are relayed by integrins, was activated in parental GEC on collagen (Fig. 4A). FAK protein expression was also enhanced on collagen, suggesting that increased synthesis or decreased degradation of FAK protein may contribute to the regulation of activity. Stable expression of a constitutively active FAK (CD2-FAK) supplanted the effect of collagen in facilitating GEC survival (Figs. 2A and 6), demonstrating features of apoptosis (Figs. 1 and 2). It should be noted that the proapoptotic effect of plastic was not due to the inability of GEC to attach and spread on plastic. Surprisingly, adhesion of GEC to laminin did not facilitate survival (Fig. 2D), even though GEC express laminin-binding cell surface receptors (1, 10, 11). Cultured GEC adhere to collagen I, collagen IV, laminin, and fibronectin and show greater adherence to the collagens, compared with the other substratum (1, 11). Anti-β1-integrin antibody significantly inhibited adhesion to ECM proteins (1, 11). Thus the antiapoptotic effect of collagen may be related to the greater efficiency of GEC adhesion to this ECM protein.

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**Fig. 5. Expression of constitutively active FAK and MEK.** Parental (Par) GEC on COL were stably cotransfected with a neomycin resistance gene and constitutively active FAK (CD2-FAK; 160 kDa). A: representative GEC clones that stably express CD2-FAK. Equal amounts of total protein were loaded into each lane. B: lysates of parental GEC on COL or PL (24 h after plating) or of GEC stably transfected with CD2-FAK (clone 213; on PL) were immunoprecipitated with antibody to FAK and immunoblotted with GST-Grb2 and anti-Grb2 antibody (left) or antibodies to phosphotyrosine (PY; middle) or FAK (right). CD2-FAK is tyrosine phosphorylated. GST-Grb2 binds to tyrosine-phosphorylated FAK and CD2-FAK. C: representative GEC clones that stably express R4F-MEK. D: ERK phosphorylation in parental GEC on COL and 3 clones of GEC that stably express R4F-MEK on PL in the absence of EGF (immunoblot with antibody to phospho-ERK). E: parental GEC were plated onto COL; R4F-MEK-expressing GEC (clone 16) and CD2-FAK-expressing GEC (clones 23 and 213) were plated onto PL for 24 h in SP medium. Then, GEC were treated with (+) or without (−) EGF (100 ng/ml) for 30 min. Equal amounts of total protein were loaded into each lane before immunoblotting with anti-phospho-ERK antibody. Both R4F-MEK- and CD2-FAK-expressing GEC showed higher levels of ERK phosphorylation compared with GEC on COL (in the absence of EGF). EGF further stimulated ERK phosphorylation in all cell lines.

**Fig. 6. Inhibition of the ERK pathway increases apoptosis.** Parental GEC on collagen (Par-COL) or plastic (Par-PL), GEC that express R4F-MEK (clone 16), and GEC that express CD2-FAK (clone 213) were plated in SP medium with or without the MEK inhibitor (PD-98059; 75 μM) or the phosphatidylinositol 3-kinase (PI3K) inhibitor (LY-294002; 10 μM) for 24 h. Apoptosis was monitored as H-33342-positive nuclei. *P < 0.01 Par-COL vs. Par-PL, R4F-MEK, and CD2-FAK. **P < 0.001 Par-COL PD-98059 vs. untreated. ***P < 0.001 R4F-MEK PD-98059 vs. untreated. ++P < 0.01 CD2-FAK PD-98059 vs. untreated. +++P < 0.001 CD2-FAK LY-294002 vs. untreated (5–6 experiments performed in duplicate).
ERK phosphorylation (Fig. 5) indicates that transmission of signals from collagen may occur via FAK and the ERK pathway. Adhesion to collagen induced small increases in ERK phosphorylation, whereas further increases occurred with EGF. B: densitometric quantification of EGF-induced ERK phosphorylation in parental GEC, as well as R4F-MEK- and CD2-FAK-expressing GEC (also see representative immunoblot in Fig. 5E). *P < 0.001 EGF vs. no EGF (4–8 experiments). C: parental GEC were plated onto collagen (C) or plastic (P) and 3 clones of R4F-MEK-transfected GEC (MEK) were plated onto plastic (~50,000 cells/well). GEC were cultured with or without EGF (100 ng/ml) in SP medium. Cell number was determined at 72 h by visual counting. *P < 0.002 EGF vs. no EGF. There were no significant differences among the groups of cells cultured without EGF (7 experiments). D: parental GEC on collagen and 2 clones of CD2-FAK-transfected GEC on plastic were plated at ~50,000 cells/well and cultured in growth factor-replete K1 medium or SP medium. Cell number was determined at 72 h by visual counting. *P < 0.02 K1 vs. SP (3–5 experiments).

whereas inhibition of Src reduced FAK and ERK phosphorylation in parallel (Fig. 4, C and E). Together, these results imply that transmission of signals from collagen may occur via FAK and the ERK pathway.

Evidence for a functional role of the Ras-ERK pathway in blocking apoptosis was provided by stable transfection of constitutively active MEK (R4F-MEK). R4F-MEK increased ERK phosphorylation (Fig. 5E) and facilitated survival (Fig. 6). Conversely, inhibition of the ERK pathway with PD-98059 increased apoptosis in parental GEC on collagen, as well as in R4F-MEK- and CD2-FAK-expressing GEC clones (Fig. 6).

Moreover, expression of the J25 Ras mutant, which blocked the ERK pathway by dominant inhibition of Ras (Fig. 8B), blocked GEC survival (Fig. 8B). Together, these results confirm the importance of the ERK pathway in mediating the survival signals emanating from collagen. By analogy to GEC, fibronectin rescued a carcinoma cell line from serum deprivation-induced apoptosis through the ERK pathway, and cell survival on fibronectin was significantly reduced by treatment with PD-98059, or by expression of a dominant-negative mutant of MEK1 (23). In other cells, survival appears to be dependent on the activation of the PI3K-Akt pathway (20), or a combination of PI3K and ERK (16). In GEC, inhibition of the PI3K pathway with LY-294002 significantly increased the level of apoptosis only in the CD2-FAK-expressing clones, but not in parental cells on collagen or in GEC that express R4F-MEK (Fig. 6). Furthermore, the J25 Ras mutant, which constitutively activates the PI3K pathway, did not facilitate survival, suggesting that activation of PI3K downstream of Ras is insufficient to supplant signals from collagen. Thus the ERK cascade appears to be the major pathway that mediates the survival signal emanating from collagen.

There are two major apoptotic pathways in mammalian cells, the death domain receptor pathway, which leads to activation of caspase-8, and the mitochondrial pathway, linked to caspase-9 (38). Both caspase-8 and -9 activities were increased that mediates the survival signal emanating from collagen.
increased in GEC adherent to plastic compared with collagen (Fig. 3A), but inhibition of caspase-9 inhibited caspase-8 in parallel. This result suggests that activation of caspase-9 may have been responsible for secondary activation of caspase-8 (43). Alternatively, a possible role for death domains in triggering anoikis, perhaps by self-association of death domain receptors or interaction with endogenous death ligands, has been proposed (20). Another explanation may be that caspase-8 was activated as a result of nonligated integrins, as recently shown in T24E carcinoma cells (45). Involvement of the mitochondrial pathway in GEC apoptosis was further supported by changes in the expression of Bax and Bcl-XL, which exert some of their functional effects on mitochondria (38). There was a significant increase in the proapoptotic protein Bax and a significant decrease in the anti-apoptotic protein Bcl-XL in GEC plated onto plastic substratum (Fig. 3B). Together, these results substantiate an antiapoptotic or survival-promoting effect of collagen. The precise interplay among ECM, Bcl-2 family members, and caspases will require further study.

In earlier studies, we demonstrated that EGF stimulated ERK activation and proliferation of cultured GEC only when the cells were adherent to collagen matrices and that proliferation was abolished by PD-98059 (12, 13). GEC adherent to laminin were unable to proliferate (9). In addition, stable transfection of a constitutively active mutant of Ras (V12Ras) supplanted the requirement for collagen, but not for growth factors (14). These results imply that EGF most likely activated the ERK pathway through endogenous Ras, whereas V12Ras had activated a separate ERK pathway. In the present study, expression of constitutively active mutants of MEK or FAK did not induce GEC proliferation independently of growth factors (Fig. 7, C and D). However, by analogy to V12Ras,
target may be in the nucleus. Induced ERK activation may occur near the receptor and the ERK may be cytosolic or nuclear, whereas growth factor-induced collagen might occur at focal adhesions and the target of pathways in GEC. For example, activation of ERK by ECM enabled EGF to induce ERK transduction was dependent on adhesion to ECM and an intact cytoskeleton (2). ECM enabled EGF to induce ERK transduction in normal glomeruli, and expression and activation were increased in PAN (Figs. 10, B–D, and 11). These results are in keeping with the view that integrin signaling was amplified in PAN. PAN did not enhance ERK phosphorylation in cultured GEC, and it is unlikely that changes in ERK phosphorylation in vivo were due to a direct effect of this compound. These results contrast with findings in mesangial cells adherent to collagen gels, where PAN enhanced tyrosine phosphorylation, including phosphorylation of FAK (49). Based on our observation that adhesion to collagen limits PAN-induced GEC injury in culture (Fig. 9), the role of enhanced ECM signaling in PAN (including activation of FAK and ERK) may be to limit apoptosis of GEC and development of sclerosis. Under resting conditions, a weaker signal from ECM/integrins may be sufficient to block apoptosis. We cannot exclude activation of FAK by growth factors (e.g., EGF) in PAN, but we did not observe EGF-R activation in acute PAN (Fig. 10E), and growth factors could not activate FAK in cultured GEC. EGF-R activation might occur during the chronic phase of PAN, when there is increased expression of heparin-binding EGF mRNA (putative ligand for EGF-R in vivo) and evidence for cell proliferation (37). Our in vivo results support the pathophysiologic relevance of the signaling pathways characterized in cultured GEC. However, further studies, including development of animal models where glomerular signaling pathways are disrupted, will be required to address the functional role of these signals in the pathophysiology of GEC injury in vivo.

To verify that signals in cultured GEC are relevant to GEC in vivo, we studied PAN, an experimental model of GEC injury that has been employed to obtain a better understanding of FSGS pathophysiology (4, 5, 36, 46). In PAN, GEC foot processes are effaced, such that a greater area of the cell surface is in contact with the glomerular basement membrane. Furthermore, expression of \( \alpha_\text{III} \) and \( \beta_\text{1} \)-integrin subunits, as well as paxillin (a FAK-interacting protein), was upregulated in early PAN (29, 32, 44), and increases in laminin and types I and IV collagen were evident as the lesion progressed (18). Integrin-mediated signals may be amplified by increased integrin expression or engagement by ECM (e.g., due to increased amounts of ECM proteins or adhesivity) (3). Consequently, it is reasonable to propose that in PAN, there may be enhanced signaling from the ECM to GEC (via \( \beta_\text{1} \)-integrins). In keeping with the earlier report (32), we demonstrate that glomerular \( \beta_\text{1} \)-integrin expression was increased in rats with PAN compared with controls (Fig. 10A). FAK, c-Src, and ERK protein expression and phosphorylation were detected at relatively low levels in normal glomeruli, and expression and activation were increased in PAN (Figs. 10, B–D, and 11). These results are in keeping with the view that integrin signaling was amplified in PAN. PAN did not enhance ERK phosphorylation in cultured GEC, and it is unlikely that changes in ERK phosphorylation in vivo were due to a direct effect of this compound. These results contrast with findings in mesangial cells adherent to collagen gels, where PAN enhanced tyrosine phosphorylation, including phosphorylation of FAK (49). Based on our observation that adhesion to collagen limits PAN-induced GEC injury in culture (Fig. 9), the role of enhanced ECM signaling in PAN (including activation of FAK and ERK) may be to limit apoptosis of GEC and development of sclerosis. Under resting conditions, a weaker signal from ECM/integrins may be sufficient to block apoptosis. We cannot exclude activation of FAK by growth factors (e.g., EGF) in PAN, but we did not observe EGF-R activation in acute PAN (Fig. 10E), and growth factors could not activate FAK in cultured GEC. EGF-R activation might occur during the chronic phase of PAN, when there is increased expression of heparin-binding EGF mRNA (putative ligand for EGF-R in vivo) and evidence for cell proliferation (37). Our in vivo results support the pathophysiologic relevance of the signaling pathways characterized in cultured GEC. However, further studies, including development of animal models where glomerular signaling pathways are disrupted, will be required to address the functional role of these signals in the pathophysiology of GEC injury in vivo.

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Fig. 11. Phospho-ERK in glomeruli of rats with PAN. A: glomerulus shows specific staining for phospho-ERK with focal and patchy accentuation. B: higher-power view of a portion of a glomerulus showing staining of glomerular capillary loops in the periphery of a glomerulus (arrowheads) adjacent to Bowman’s space (B). The asterisks denote capillary lumina. C: negative control (i.e., incubation with nonimmune rabbit IgG). Tubular staining adjacent to the glomeruli (seen in all panels) is nonspecific. Bars = 10 μm.

R4F-MEK or CD2-FAK enabled exogenously added growth factors to further stimulate ERK phosphorylation above resting levels (Fig. 7B) and to induce proliferation of GEC in the absence of collagen (Fig. 7, C and D). These experiments further support the view that constitutively active FAK and MEK mutants supplant ERK activation by collagen, whereas growth factor-induced ERK activation occurs via a distinct Ras-ERK pathway. Moreover, a survival signal from collagen, or from constitutively active protein kinases that supplant this collagen effect, is a prerequisite for EGF-dependent activation of EGF-R and the ERK pathway as well as for proliferation. In fibroblasts, EGF-dependent ERK activation was dependent on adhesion to ECM and an intact cytoskeleton (2). ECM enabled EGF to induce ERK translocation to the nucleus and phosphorylation of transcription factors, whereas EGF was ineffective in suspended cells. Further studies will be required to characterize the ERK pathways in GEC. For example, activation of ERK by collagen might occur at focal adhesions and the target of ERK may be cytosolic or nuclear, whereas growth factor-induced ERK activation may occur near the receptor and the target may be in the nucleus.

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