

# Differential Regulation of Soluble Guanylyl Cyclase Expression and Signaling by Collagens: Involvement of Integrin-Linked Kinase

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Glomerular diseases are characterized by an abnormal synthesis of extracellular matrix proteins, such as collagen type I. Evidence that growth on collagen type I downregulates soluble guanylyl cyclase (sGC) expression and the responsiveness of human mesangial cells to nitric oxide (NO) by activating specific integrin signals involving integrin-linked kinase (ILK) is presented. Human mesangial cells were grown on collagen type I or IV for 24 to 72 h. Compared with collagen IV, growth on collagen I reduced the protein expression and NO-stimulated enzyme activity of sGC. This downregulation was effected at the level of transcription, because steady-state sGC mRNA expression was reduced on collagen I, but inhibition of transcription with Actinomycin D revealed no differences in transcript stability between the two culture conditions. Collagen I also reduced the capacity of cells to relax in response to NO after H<sub>2</sub>O<sub>2</sub> challenge and inhibited NO-induced phosphorylation of vasodilator-activated phosphoprotein, a target of cyclic guanine monophosphate-dependent protein kinase. Examination of the surface expression of integrins, the receptors for extracellular matrix components, revealed that  $\alpha$ 1 and  $\alpha$ 2 integrin subunits were more abundant on cells that were grown on collagen IV and that surface expression of  $\beta$ 1 integrin did not vary with collagen type. However, growth on collagen I induced  $\beta$ 1 integrin to adopt its active conformation, and this activation of  $\beta$ 1 integrin was accompanied by increased activity of its downstream effector ILK. Dominant-negative suppression of ILK signaling relieved the suppression of sGC expression and NO-induced vasodilator-activated phosphoprotein phosphorylation induced by collagen I.

**M**esangial dysfunction is associated with several renal diseases, such as glomerulonephritis and diabetes, and a hallmark of these disorders is an abnormal remodeling of the extracellular matrix (ECM). Normal ECM components such as fibronectin (FN), laminin, and collagen type IV are quantitatively altered, and unusual components, such as collagens type I and III, appear (1,2).

The ECM transmits its signals to cells through interactions with members of the integrin superfamily of transmembrane receptors (3), which directly activate many intracellular signaling events. Important among these events is the activation of the tyrosine kinase focal adhesion kinase (FAK) upon interaction with the cytoplasmic tail of  $\beta$ 1 integrins (4) and the activation of the serine/threonine kinase integrin-linked kinase (ILK) by interaction with multiple integrins (e.g.,  $\beta$ 1 and  $\beta$ 3

integrins) and cytoskeleton-associated proteins (5). ILK mediates FN matrix deposition in diabetic glomeruli (6) and the secretion by glomerular mesangial cells (MC) of TGF- $\beta$ , a key regulator of the synthesis and degradation of ECM components (7). Increased expression of ECM components has been reported to induce expression of the corresponding integrin receptors in several glomerular diseases, thus linking altered cell-ECM interaction to the progression of renal disease (8).

Most chronic renal diseases are characterized by decreased activity of the nitric oxide (NO) system (9), and NO is an important regulator of renal or vascular ECM remodeling (10,11). NO, generated either by endothelial cells or MC, participates in the regulation of the glomerular microcirculation by modifying the tone of the afferent arteriole and MC (12), principally *via* activation of soluble guanylyl cyclase (sGC) and subsequent stimulation of guanosine monophosphate (cGMP)-dependent protein kinase (PKG) (13). Chronic NO insufficiency causes hypertension, atherosclerosis, and glomerular damage (14–16), and increased NO production may be involved in the early pathogenic hemodynamic changes in diabetes (17). In relation to the ECM, NO attenuates the activity of matrix metalloproteinase-9 an ECM-degrading enzyme on rat MC (18), whereas NO deficiency

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increases matrix metalloproteinase-9 activity, contributing to matrix degradation (19).

It has been proposed that an altered composition of the ECM surrounding the MC or in the capillary walls could be responsible for the decreased activity of the NO system detected in most chronic renal diseases (20). In a previous study, we demonstrated that NO synthesis is decreased in human endothelial cells that are cultured on collagen type I (COL I), and we further showed that this decrease is due to the downregulation of endothelial NO synthase expression, which was mediated by ILK (21). In this study, we hypothesized that the interaction of MC with the altered ECM generated upon glomerular injury might differentially regulate sGC expression and therefore the biologic responses of these cells to NO. We propose that this pathway suggests a novel model for the role of ECM–MC interactions in the progression of chronic renal disease.

## Materials and Methods

Collagen types I and IV, FN, poly-L-lysine (PolyL), H<sub>2</sub>O<sub>2</sub>, collagenase,  $\beta$ -tubulin antibody, sodium nitroprusside (SNP), and actinomycin D (AD) were purchased from the Sigma Chemical Company (St. Louis, MO). sGC- $\beta$  antibody was purchased from Calbiochem (La Jolla, CA). Spermine-NONOate (spNO) was purchased from Alexis Biochemicals (Carlsbad, CA). Peroxidase-conjugated goat anti-rabbit IgG and anti-integrin antibodies (anti  $\alpha$ 2, anti- $\alpha$ v $\beta$ 3, anti- $\alpha$ v, anti- $\beta$ 1, and anti- $\beta$ 3 active conformation) were purchased from Chemicon (Temecula, CA). Anti- $\alpha$ 1 antibody was from Immunotech (Cedex, France). RPMI 1640, FCS, and antibiotics were purchased from Bio Media (Bous-sens, France). ILK antibody was from Upstate Biotechnology (Lake Placid, NY).  $\alpha$ -[<sup>32</sup>P]-dCTP, RediPrime radiolabeling system, Hybond-N membrane, and the cGMP RIA kit were purchased from Amersham Biosciences (Piscataway, NJ).

### Human MC Culture

Human mesangial cells (HMC) were cultured according to previously described procedures (22). The identity of the cells was confirmed by morphologic and functional criteria, as described previously (23). When cells reached confluence, they were subcultured at a ratio of 1:4 in RPMI 1640 supplemented with 10% FCS and antibiotics. The cells were serum-deprived for 24 h before the treatments.

### Experimental Design

Studies were performed in cells that were cultured on a thin film of COL I or COL IV. For this, culture plates were incubated for 16 h at 4°C in a solution of 12.5 J.g/ml COL I or COL IV in bicarbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub> and 35 mM NaHCO<sub>3</sub> [pH 9]), to allow the formation of a thin film of collagen, as described (24). Then, the remaining collagen was discarded and Petri dishes were washed with Hanks' balanced salt solution to restore the pH. Trypsinized cells were seeded onto the COL I- or COL IV-coated plates at 4 X 10<sup>4</sup> cells/cm<sup>2</sup>. Cells were grown for the indicated times in RPMI 1640 supplemented with 10% FBS. In experiments in which HMC were grown for 72 h on COL I or IV, cells were serum-starved for 24 h before the cells were harvested. In some experiments, we used plates that were coated with FN at 2.5 J.g/ml or PolyL at 0.5 J.g/ml as controls.

**Transient Transfection Experiments.** HMC were cultured on COL I or COL IV for 24 h. Cells then were incubated with 2 J.g of plasmid and 4 J.l of LipofectAMINE reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). The plasmid used was pcDNA 3.1 inserted with a cDNA for His-V5-tagged, kinase-

deficient ILK (ILK-KD), provided by S. Dedhar (British Columbia Cancer Research Centre, Vancouver, British Columbia) (25). Empty vector was used as a control. Cells were harvested 48 h after transfection. Transfected cells were analyzed for cell viability by the trypan blue exclusion viability assay. In some experiments, a plasmid encoding  $\beta$ -galactosidase under the control of the cytomegalovirus promoter (pCMV- $\beta$ -gal) was used to determine transfection efficiency. Consistently, transfection efficiency was >60%.

**Protein Extraction and Western Blot Analysis.** After treatment, HMC were lysed in protein lysis buffer (21). For performing Western blot analysis, total cell extracts (30 J.g/lane) were size-fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h and then incubated with a rabbit polyclonal antibody against sGC- $\beta$ 1. After washing, blots were incubated with a peroxidase-conjugated goat anti-rabbit Ig, and specifically bound antibody was detected with the SuperSignal system (Pierce, Rockford, IL). The films then were scanned and analyzed with appropriate software (NIH Image 1.55; National Institutes of Health, Bethesda, MD). In some experiments, the blots were stripped and reblotted with an antibody against  $\beta$ -tubulin to confirm that sGC protein changes were specific.

**Northern Blot Analysis.** HMC total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (26). For the probe, a 408-bp sGC cDNA fragment was obtained by reverse transcription-PCR of HMC total RNA by using the following primers based on the human sGC- $\beta$ 1 sequence (27): Forward, base position 3505'-CGTGTCTGGGGCTCTAA-3', and reverse, base position 774 5'-AC-CACTAGGTCCCGGTC-3'. After hybridization with the sGC probe, blots were rehybridized with a [<sup>32</sup>P]-labeled probe for 18S ribosomal RNA as an internal standard for each sample. Blots were autoradiographed, and the densitometrically scanned band intensities on the exposed films were analyzed with the NIH Image 1.55 software.

**Measurement of cGMP Synthesized by sGC in HMC.** Reactions were started by the addition of an NO donor (1 J.M SNP or 1 J.M spNO). After 30 min, the medium was aspirated and ethanol extraction of cGMP was carried out as described previously (28). Concentrations of cGMP were determined with a commercial [<sup>125</sup>I]cGMP RIA kit. The protein concentrations in the pellets were determined by the BCA method.

**Immunoprecipitation and Measurement of ILK Activity.** ILK kinase activity in HMC extracts was determined by immunoprecipitation *in vitro* kinase assay (29). Briefly, 300 J.g of extracted cell proteins was immunoprecipitated with the anti-ILK antibody. Nonimmune IgG was used as the negative control in all experiments. Myelin basic protein was used as the ILK substrate, [<sup>32</sup>P] ATP was the phosphate donor, and phosphorylated proteins were electrophoresed on 12% SDS-PAGE gels. After autoradiography, the phosphorylated proteins were quantified by densitometric analysis with NIH Image software.

**Measurement of Cellular Relaxation.** Planar cell surface area (PCSA) was determined by computer-aided planimetric techniques (28). Cells were grown at low density on 20-mm plates. After 15 min of temperature equilibration, the experiments were started by preincubating the cells with 1 J.M spNO M for 5 min, at which time H<sub>2</sub>O<sub>2</sub> (0.1 mM) was subsequently added to each treatment. Cells were observed under phase contrast with an inverted photomicroscope (Olympus IMT 2, Tokyo, Japan). Microphotographs were taken before H<sub>2</sub>O<sub>2</sub> addition and 30 min after this addition.

**FACS Analysis and Immunofluorescence.** Surface expression of integrins was determined by flow cytometry using a CyAn MLE flow cytometer (DakoCytomation, Barcelona, Spain). Cells were detached from the collagen-coated plates with trypsin/EDTA and then incubated with anti-integrin antibodies at 4 J.g/ml for 1 h at 4°C. After cells were

rinsed three times with ice-cold PBS, they were incubated with a secondary antibody (anti-rabbit or anti-mouse, depending on the primary antibody) coupled to FITC or PE1/200. After three additional washes, cells were fixed with 3.7% formaldehyde and analyzed.

For testing surface expression of integrin molecules by immunofluorescence, HMC were incubated with anti-integrin antibodies, washed three times with PBS, incubated with secondary antibody coupled with FITC, and then fixed with 3.7% paraformaldehyde. Nuclei were stained with Hoechst dye. Cells were visualized by confocal microscopy (Radiance 2100; Bio-Rad, Hercules, CA).

### Statistical Analyses

Because  $n < 10$  in every case, comparisons were made using non-parametric statistical tests: The Friedman test, followed by the Wilcoxon test and the Bonferroni correction when appropriate. The level of statistically significant difference was defined as  $P < 0.05$ .

## Results

### HMC Grown on COL I Show Decreased sGC Expression and Activity

We first studied the sGC protein content of HMC that were grown on COL I and on the normal matrix/basement membrane component COL IV for 24, 48, or 72 h. The Western blot in Figure 1A shows that steady-state sGC protein expression increased in a time-dependent manner with both collagens. However, at all of the times analyzed, the expression of sGC in cells that were grown on COL I was significantly lower. This difference was maximal after 72-h contact with collagen. For confirming this result, HMC were grown on plates that were coated with FN or PolyL. The Western blot in Figure 1B shows no difference between the levels of sGC expression achieved with COL IV, FN, or PolyL. However, sGC protein expression in HMC that were grown on COL I was markedly lower.

To determine whether the different culture substrates also altered sGC activity, we next tested the effect of exogenous NO on cGMP synthesis. HMC that were grown on the two collagen types were exposed for 15 min to vehicle or to one of two different NO donors: spNO (1 J.M) or SNP (1 J.M). cGMP concentrations were determined by RIA. Figure 2 shows that sGC activity in response to NO was increased significantly above basal levels in cells that were cultured on either collagen type, but this effect was significantly less in cells that were grown on COL I.

We next performed Northern blot analysis for sGC  $\beta$  subunit mRNA to determine whether the inhibitory effect of COL I was at the level of mRNA expression. As shown in Figure 3, sGC mRNA expression increased in a time-dependent manner in cells that were grown for up to 72 h on COL IV, whereas this progressive increase was not observed in cells that were grown on COL I.

For determining whether the COL I-mediated downregulation of sGC  $\beta$  subunit mRNA expression was effected through a reduction in mRNA stability, transcription in HMC was stopped by addition of AD (10 J.M). As shown in Figure 4, no changes in sGC mRNA stability were detected in HMC that were cultured on either matrix for 24 h and then exposed to AD for 2 to 24 h. These results indicate that the suppressive effect of COL I on sGC mRNA expression involves a transcriptional effect rather than a decrease in mRNA stability.

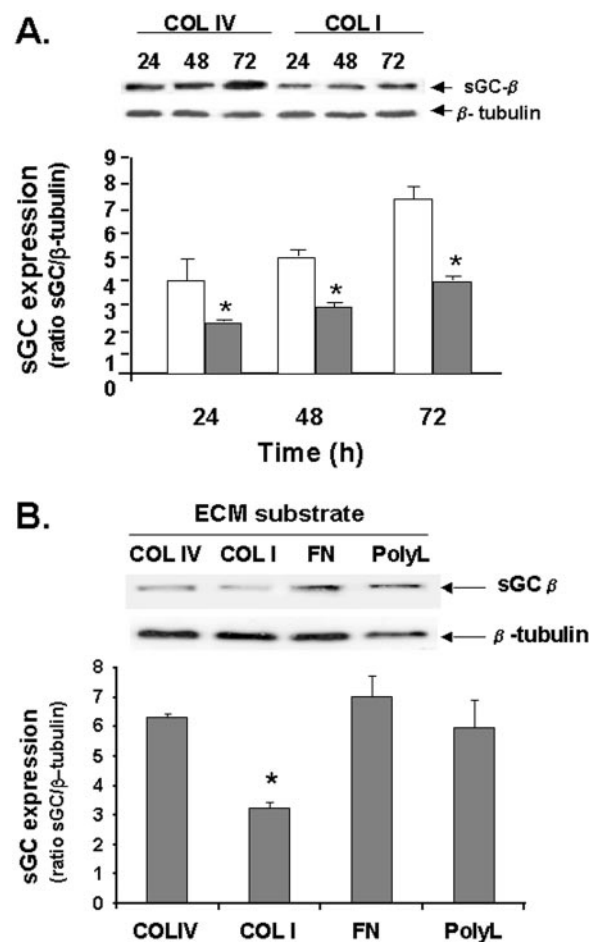
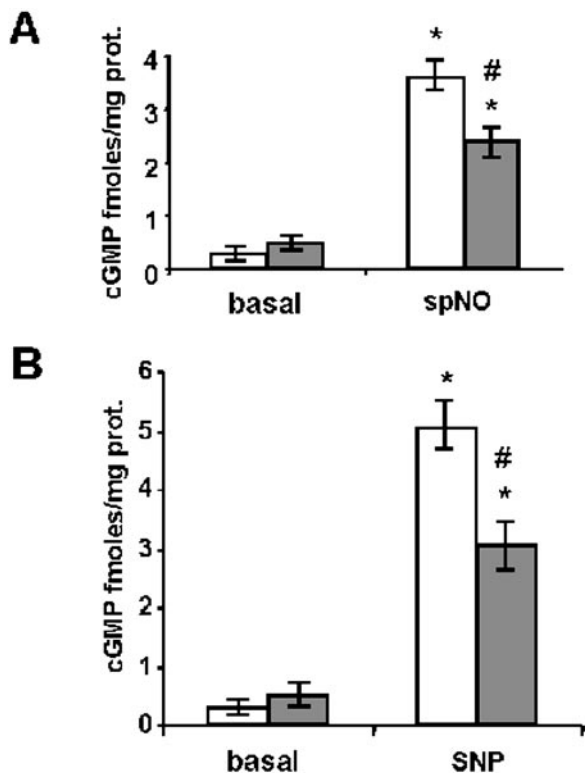


Figure 1. Steady-state expression of soluble guanylyl cyclase 13 subunit (sGC-13) protein in human mesangial cells (HMC) grown on collagen IV (COL IV) and COL I. (A) HMC were grown on COL IV or COL I for the indicated times. (Top) Representative Western blot of sGC and  $\beta$ -tubulin. (Bottom) The graph presents densitometric band analysis normalized to  $\beta$ -tubulin content, with the white and gray bars showing expression in cells grown on COL IV and COL I, respectively.  $n = 6$ ;  $*P < 0.05$  versus COL IV at the corresponding time point. (B) Western blot analysis of sGC expression in HMC grown for 72 h on COL IV, COL I, fibronectin (FN), or poly-L-lysine (PolyL). (Top) Representative Western blot of sGC and  $\beta$ -tubulin. (Bottom) The graph presents densitometric band analysis normalized to  $\beta$ -tubulin content.  $n = 6$ ;  $*P < 0.05$  versus COL IV, FN, and PolyL.

### COL I and COL IV Differentially Modulate the Relaxing Effect of NO on $H_2O_2$ -Induced Contraction

To evaluate the physiologic relevance of COL I-dependent sGC downregulation, we tested the relaxing effect of NO on HMC contraction induced by  $H_2O_2$ , which acts as a contractile agonist in several cell types, including MC (28,30–32). HMC were grown on the two collagens for 72 h, and cells then were preincubated with exogenous NO for 5 min.  $H_2O_2$  was added (time 0), and the incubation was continued for 30 min. Microphotographs were taken at times 0 and 30 min, and the PSCA was measured from these.  $H_2O_2$  induced a significant contrac-





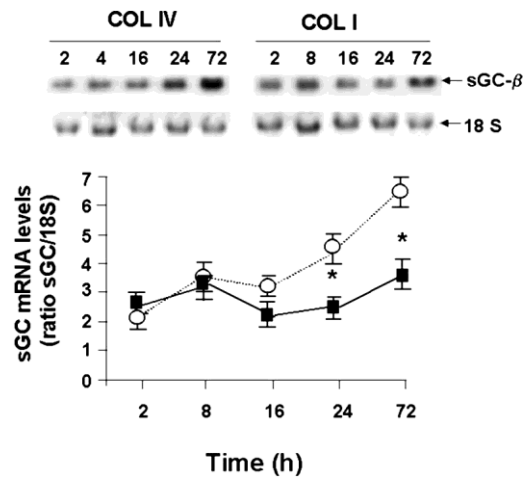
**Figure 2.** Effect of COL IV and COL I on sGC- $\beta$  activity in HMC. Cells were cultured on COL IV (O) or COL I (u) for 72 h. Cells then were activated by exposure to one of two nitric oxide (NO) donors: Spermine-NONOate (spNO; 1 J.M; A) or sodium nitroprusside (SNP; 1 J.M; B). Control cells were exposed to vehicle (basal). cGMP concentrations were determined by RIA. Data are expressed as femtomoles of cGMP per J.g of protein:  $n = 6$ ; \* $P < 0.05$  versus basal; # $P < 0.05$  versus COL IV.

tion of HMC that were cultured on COL IV, expressed as a reduction of the PCSA at 30 min, which was almost completely prevented by preincubation with the NO donor (Figure 5A). In contrast, preincubation with NO only partially prevented the H<sub>2</sub>O<sub>2</sub> contractile effect in cells that were cultured on COL I.

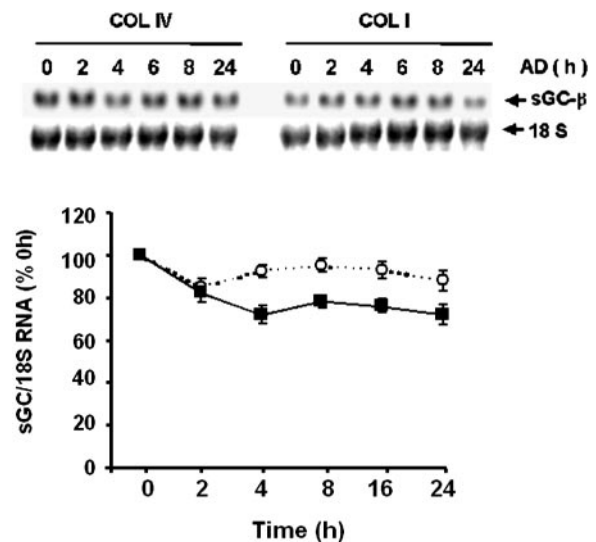
To confirm the role of COL I-induced downregulation of sGC expression and activity in MC responses to NO, we analyzed the phosphorylation of vasodilator-activated phosphoprotein (VASP), a downstream target of cGMP-dependent protein kinase (PKG). VASP phosphorylation has been established as a biologic marker of the NO/PKG activity (33). As shown in Figure 5B, VASP phosphorylation was induced in response to NO in cells that were grown in COL IV, whereas phospho-VASP levels in HMC that were grown on COL I were not increased.

#### Integrin Expression Profile Is Different in HMC Cultured on COL I and COL IV

To investigate a potential mechanism underlying the differences in the NO/sGC/cGMP pathway between HMC that are grown on collagens IV or I, we decided to determine the integrin profile of these cells by flow cytometry and confocal microscopy. The known collagen receptors are formed by the

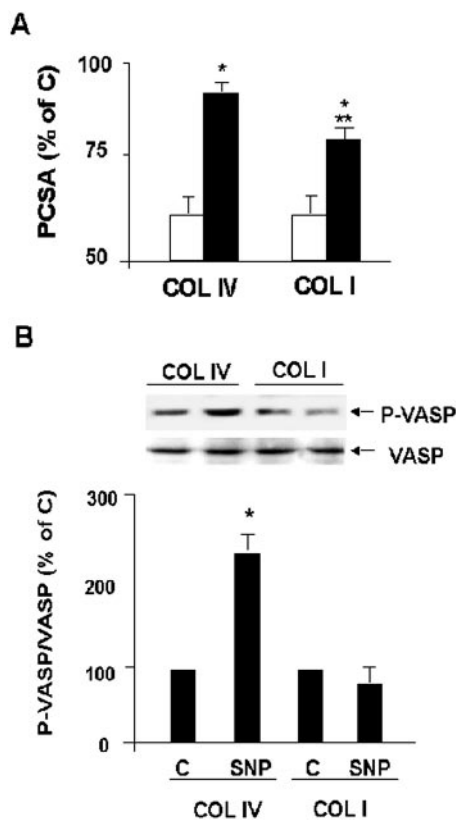


**Figure 3.** Steady-state levels of sGC- $\beta$  mRNA in HMC grown on COL IV and COL I. Northern blot analysis of the  $\beta$ 1 subunit of sGC was performed, with blotting for 18S rRNA used as a loading control. (Top) Representative Northern blots. (Bottom) Densitometric sGC mRNA band intensities are plotted from HMC grown on COL IV (E) and COL I (f), normalized to 18S rRNA content. Results are expressed in arbitrary densitometric units:  $n = 6$ ; \* $P < 0.05$  versus COL IV at the corresponding time point.



**Figure 4.** sGC- $\beta$  mRNA stability in HMC grown on COL IV and COL I. Cells were cultured on COL IV or COL I for 24 h. Cells were then treated with actinomycin D (AD; 10 J.M) for the indicated times, and Northern blot analysis of sGC expression was carried out, with blotting for 18S rRNA used as a loading control. (Top) Representative Northern blots. (Bottom) Densitometric sGC mRNA band intensities are plotted from HMC grown on COL IV (E) and COL I (f). The data are expressed as percentage of values at time = 0. Means  $\pm$  SE are shown ( $n = 6$ ).

association of the  $\beta$ 1 integrin subunit with either of the  $\alpha$ 1 and  $\alpha$ 2 integrin subunits. Our flow cytometry experiments showed that although MC express both  $\alpha$ 1 and  $\alpha$ 2 integrin subunits, the expression of these subunits was markedly higher in cells that



**Figure 5.** COL I reduces the physiologic response to NO in HMC. (A) Contractile response of HMC to  $H_2O_2$ . HMC were cultured on COL IV or COL I for 72 h and were then preincubated with spNO (1 J.M; f) or vehicle (O) for 5 min. 0.1 mM  $H_2O_2$  was then added, and the cells incubated for another 30 min. Microphotographs were taken at the beginning (time 0) and the end of each experiment (time 30), and planar cell surface area (PCSA) was measured from these. The results are expressed as the percentage of cell area at time 0 remaining at time 30 (% of C). Data are the means  $\pm$  SE of six independent experiments. \* $P < 0.05$  versus vehicle; \*\* $P < 0.05$  versus spNO in COL IV. (B) NO-induced phosphorylation of vasodilator-activated phosphoprotein (VASP). HMC were grown on COL IV or COL I for 72 h and then treated for 30 min with SNP (1 J.M) or vehicle (C). (Top) Representative Western blots comparing the expression of phosphorylated VASP (P-VASP) and total VASP. (Bottom) The graph presents densitometric band analysis of P-VASP content expressed as the percentage of the value for non-NO-exposed cells (% of C). Data are the means  $\pm$  SE of six independent experiments. \* $P < 0.05$  versus C in COL IV and C and SNP in COL I.

were grown on collagen IV than in those that were grown in collagen I (Figure 6A). The expression of  $\beta_1$  integrin subunits was comparable between HMC that were grown on both collagen types. These findings were confirmed by examination of immunofluorescence-stained cells by confocal microscopy (Figure 6B). Confocal microscopy furthermore demonstrated that the expression of  $\alpha_v$  and  $\alpha_v\beta_3$  integrins was also higher in HMC that were grown on collagen type IV (Figure 6B). The expression of  $\alpha_v$  and  $\beta_3$  integrin subunits was also higher in

collagen type IV as revealed by flow cytometry (av:  $47.10 \pm 0.9\%$  in COL IV versus  $21.6 \pm 1.03\%$  in COL I;  $\beta_3$ :  $23.49 \pm 0.5\%$  in COL IV versus  $12.52 \pm 0.89\%$  in COL I).

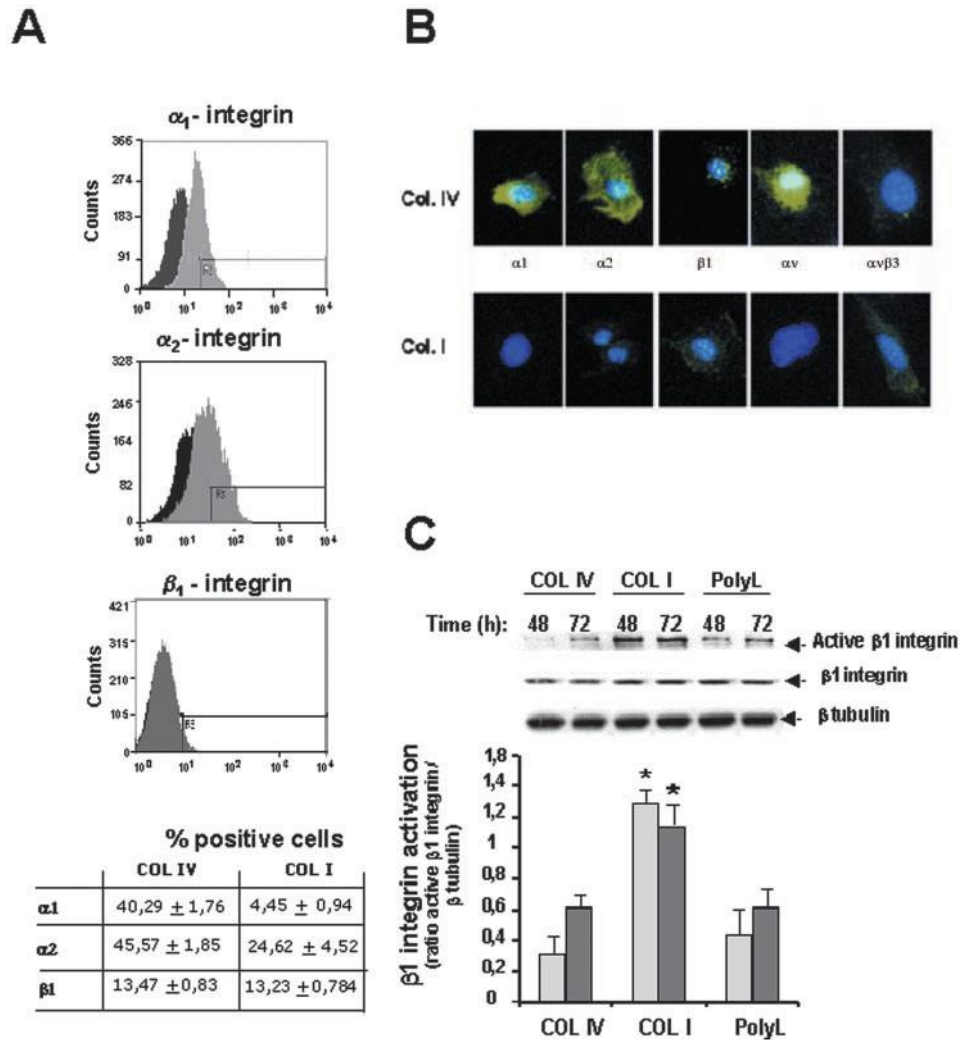
Interaction of  $\beta_1$  integrin with its ligand induces a conformational switch that is often linked to the initiation of a signaling cascade involving ILK (5). This conformational change exposes the activation epitope of  $\beta_1$  integrin, which can be detected with an antibody specific for the active conformation of human  $\beta_1$  integrins. As shown in Figure 6C, the expression of the activation epitope of  $\beta_1$  integrin is higher in HMC that were grown on COL I for 48 and 72 h compared with cells that were grown on either COL IV or PolyL, in which  $\beta_1$  integrin protein expression was relatively unchanged. The result furthermore was confirmed by flow cytometry, which showed that only  $17.18 \pm 1.2\%$  of the HMC that were grown in COL IV expressed the activation epitope of  $\beta_1$  integrin compared with  $51.95 \pm 0.7\%$  of the HMC that were grown in COL I. COL I thus promotes a different integrin expression profile and activates  $\beta_1$  integrin, potentially triggering different signaling pathways.

#### *ILK Plays a Role in Decreased sGC Activity Observed in Cells Grown on COL I*

ILK is involved in glomerular matrix deposition and growth factor secretion. We next investigated the potential role of ECM-mediated activation of ILK in the reduced expression of sGC that we observed in HMC that were grown on COL I. HMC were grown on COL IV or I for 24 h and then transfected with a dominant negative form of ILK (ILK-KD) or an empty plasmid used as a control (pcDNA 3.1; C). The expression of sGC protein was determined 48 h later and is presented in Figure 7A. In cells that were transfected with empty plasmid, we observed the same lowered sGC protein expression in cells that were grown on collagen I as observed earlier in nontransfected cells. However, in HMC that were transfected with the ILK-KD construct, the inhibitory effect of collagen type I was completely blocked. The expression of sGC in cells that were grown on COL IV was also moderately increased by transfection with ILK-KD.

To evaluate the importance of ILK as a mediator of this effect, we measured the *in vitro* ILK activity, using myelin basic protein as a substrate (Figure 7B). These experiments showed that endogenous ILK activity was higher in cells that were grown on COL I compared with those that were grown on COL IV and confirmed that transfection with the ILK-KD construct blocked ILK activity in both COL IV- and COL I-cultured HMC.

These results suggest that ILK might participate in the regulation of NO synthesis by downregulating sGC expression. To explore further the role of ILK in sGC expression in both collagen types, we measured the effect of ILK-KD on the phosphorylation of VASP. Figure 7C shows that VASP phosphorylation was increased in response to NO in cells that were grown on COL IV whether these cells were transfected with pcDNA3.1 or ILK-KD. This contrasted markedly with the situation in HMC that were grown on COL I. These cells that were transfected with pcDNA 3.1 showed little or no response to NO. However, transfection of COL I-grown HMC with ILK-KD overcame the COL I-mediated suppression of NO-induced



**Figure 6.** Integrin profile in HMC grown in COL IV and I. HMC were grown on COL IV- or COL I-coated plates for 72 h. (A) Cell-surface expression of integrins determined by flow cytometry. Light gray peaks correspond to integrin expression in HMC grown on COL IV, and dark gray peaks correspond to HMC grown on COL I. The table shows the percentages of integrin-positive cells for each substrate ( $\pm$ SEM,  $n = 4$ ). (B) Confocal immunofluorescence of the expression of  $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ , and  $\alpha v$  integrin subunits and  $\alpha v\beta 3$  integrin. The green staining (FITC) corresponds to integrin expression, and blue (Hoechst) corresponds to nuclei. The images show representative cell staining from one of four independent experiments. Magnification, X63. (C) Activation of  $\beta 1$  integrin by different substrates. HMC were grown on COL IV, COL I, or PolyL for 48 or 72 h, and the expression of active  $\beta 1$  integrin was detected by Western blot. The upper panel shows representative blots of the expression of LIBS (active  $\beta 1$  integrin), total  $\beta 1$  integrin, and  $\beta$ -tubulin (a control for equal protein loading). The graph in the lower panel presents densitometric band analysis of the content of active  $\beta 1$  integrin. Light gray bars are 48-h culture on substrate; dark gray bars are 72 h. Data are the means  $\pm$  SE of six independent experiments.  $*P < 0.05$  versus COL IV.

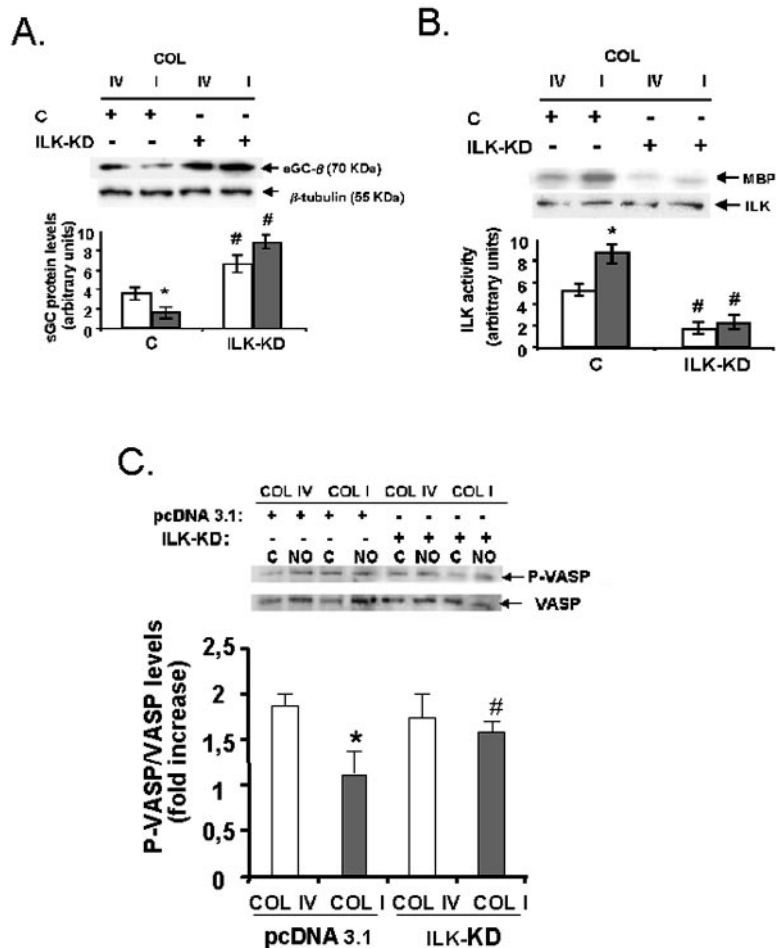
VASP phosphorylation. These results support a role for ILK in mediating the observed effects of COL I on sGC expression and NO responsiveness.

## Discussion

The data presented here provide a clear demonstration of the role of ECM composition in the regulation of sGC expression and activity. Our results show that growth on COL I diminishes sGC in HMC by downregulating the transcription of sGC mRNA. These decreases were accompanied by impaired responses to NO of sGC activity, cell relaxation, and PKG-mediated VASP phosphorylation. These parameters of NO respon-

siveness were unaffected in HMC that were cultured on COL IV. To our knowledge, this study is the first to demonstrate such a modulation of sGC activity by an ECM component. We furthermore have provided evidence for a mechanism underlying this process, involving the differential activation of  $\beta 1$  integrins, leading to the specific activation by COL I of ILK. These findings highlight the importance of altered ECM-integrin interactions in the regulation of the NO/sGC/cGMP pathway and support an important role for ILK in the development of chronic renal disease.

In glomerular diseases, MC undergo phenotypic changes that are marked by an increase in their proliferative capacity



**Figure 7.** The effect of kinase-deficient integrin-linked kinase (ILK-KD) on steady-state sGC-13 protein expression, ILK activity, and VASP phosphorylation in HMC grown on COL IV and COL I. HMC were transiently transfected with either pcDNA 3.1 control plasmid (C) or a plasmid encoding ILK-KD. Transfected cells were cultured over COL IV (O) or COL I (u) for 72 h (transfection efficiency approximately 60%). (A) Western blot analysis of sGC protein. (Top) Representative Western blot. (Bottom) Densitometric band analysis of sGC expression.  $n = 6$ ;  $*P < 0.05$  versus COL IV (C);  $*P < 0.05$  versus corresponding collagen control (C). (B) *In vitro* kinase activity of ILK. ILK activity was determined in lysates of transfected cells, with myelin basic protein (MBP) as substrate, and MBP autoradiograph band intensities were normalized to total ILK expression measured by Western blot (ILK). (Top) Representative ILK kinase assay. (Bottom) Densitometric band analysis of MBP phosphorylation.  $n = 6$ ;  $*P < 0.05$  versus COL IV (C);  $*P < 0.05$  versus COL I (C). (C) Western blot analysis of VASP phosphorylation. Transfected cells were stimulated with SNP (1 J.M) for 30 min (NO) or with vehicle (C). The upper panel shows representative Western blots and the graph in the lower panel presents the densitometric band analysis of VASP phosphorylation expressed as the fold increase with respect to unstimulated cells.  $n = 6$ ;  $*P < 0.05$  versus COL IV (pcDNA 3.1);  $*P < 0.05$  versus COL I (pcDNA 3.1).

and the active secretion of ECM. This latter process involves the *de novo* synthesis of COL I and the deposition of interstitial collagens (2). COL I has been shown to alter the contractile response of MC to many vasoactive factors. Miralem *et al.* (34) demonstrated that cells that were grown on COL I were more contractile in response to endothelin with a more rapid myosin light chain-kinase-dependent phosphorylation of myosin light chain. Our previous work furthermore demonstrated that growth of HMC on COL I elicits a significant contraction in response to  $H_2O_2$  (24). However, disturbance of the NO/sGC/cGMP system has been proposed previously as an important mechanism through which the ECM modulates MC tone *in*

NO synthesis is reduced in human endothelial cells that were cultured on COL I (21). Our study clearly shows that COL I regulates the tone of HMC by altering their ability to relax in response to NO. This effect may be explained by the failure of HMC that were grown on COL I to increase their cGMP production in response to NO as a result of the downregulation of sGC.

The mechanism through which COL I and IV differentially modulate sGC expression must involve changes in sGC promoter activity because mRNA stability remained unchanged in both culture conditions. Several treatments have been reported to decrease the rate of sGC transcription: Prolonged NO (36),



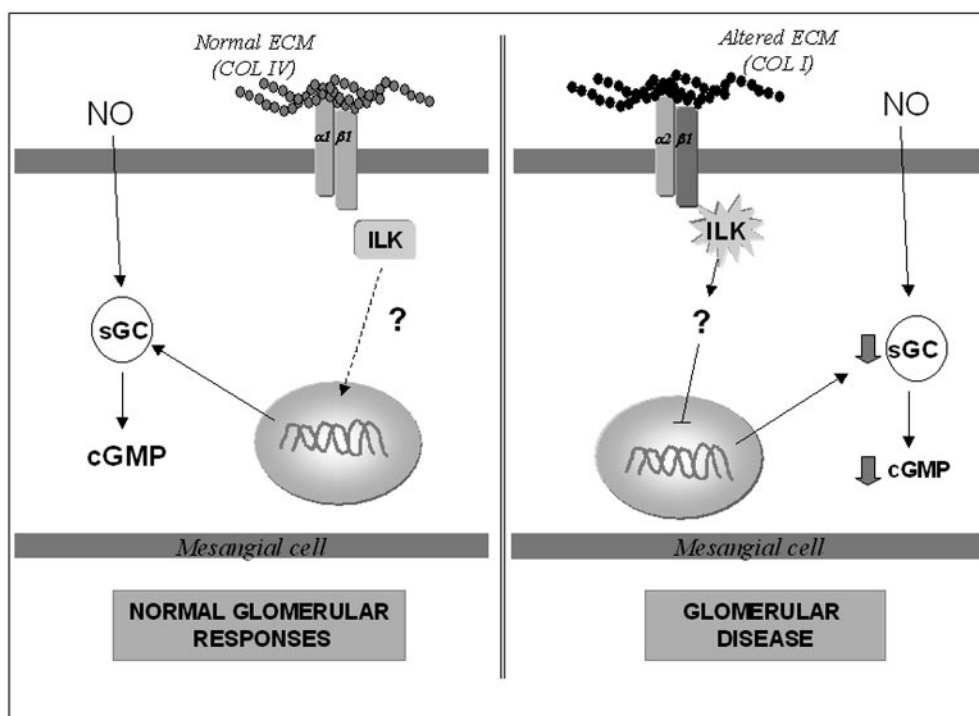


Figure 8. Proposed model for the effects of COL I on sGC expression. We propose a model in which altered extracellular matrix (ECM) proteins that are produced after glomeruli injury such as COL I would make contact with different integrins (*i.e.*,  $\beta 1$  integrin), activating them and triggering the ILK signaling pathway. ILK signaling would be responsible in part for the downregulation of sGC mRNA and protein and the resulting impaired response to NO. Decreased levels of sGC would produce less cGMP in response to NO, which in turn would contribute to the progression of glomerular disease. By contrast, normal ECM proteins such as COL IV will not activate this signaling pathway, thus ensuring normal levels of sGC and a maintenance of glomerular responses to NO/cGMP.

IL-1 (39), and nerve growth factor (40). However, relatively little is known about the regulation of the promoter regions of mammalian sGC (41). Recently, Sharina *et al.* (42) demonstrated the regulation of the proximal promoter of the  $\beta 3$  subunit of sGC by Sp-1, Gata, and CCAAT-binding factor transcription factors. Further studies are needed to elucidate the transcription factor or factors involved in the effects observed.

That the majority of cell responses elicited by ECM depend on integrin binding led us to speculate that integrins are responsible for the downregulation of sGC in HMC that are grown on COL I. Our data confirm that mesangial cells that are grown on COL IV and I express the same collagen-binding integrins but at different levels. Whereas cells that are grown on COL IV show higher expression of integrins  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha v$  or  $\alpha v \beta 3$ , cells that are grown on both collagen types express  $\beta 1$  integrin at the same level. However, the activation of  $\beta 1$  integrin is evidently different in cells that are grown on each collagen type. Interesting in this regard are recent reports showing the importance of  $\alpha$  integrin subunit expression in collagen-specific responses (43,44).

A key downstream mediator in integrin signaling is ILK (45). The ILK pathway formed the focus of our attention because this kinase has been shown to be involved in some differential responses elicited by COL I compared with COL IV. ILK regulates the transcription (935) of the endothelial sGC promoter that

endothelial cells that are grown on COL I (2), and the increased ILK activity in HMC that are grown on COL I stimulates TGF- $\beta 3$  synthesis (7). Our results clearly show that ILK activity is augmented by growth of HMC on COL I and that blockade of ILK activity reverses the downregulation of sGC expression.

It is possible that the differences observed in ILK activity may be dependent on the different  $\beta 1$  integrin activation by collagens. The ability of ILK to activate gene expression has been demonstrated previously: ILK can regulate the activity of transcription factors such as  $\beta 3$ -catenin-T cell/lymphoid enhancer factor (46), AP-1 (29), CREB (47), and NF- $\kappa$ B (48). Thus, ILK could regulate sGC expression by activating/inhibiting some of these transcription factors directly. Another possibility is that COL I and COL IV differentially activate additional signaling pathways that modulate sGC promoter activity.

Accumulation of glomerular ECM is a characteristic hallmark of progressive renal disease. On the basis of our results (summarized in Figure 8), it can be hypothesized that the contact of MC from injured glomeruli with altered ECM proteins could change the synthetic phenotype of these cells, leading to a decrease in sGC expression. This decrease would be dependent on ECM-integrin interaction and ILK activity, and the decreased sGC expression would render them unable to respond to NO, further contributing to the glomerular damage. AMP and cGMP elevating agents (97,98), endothelin and/or

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*vitro* and *in vivo* (9,35), and our group has demonstrated that cAMP- and cGMP-elevating agents (37,38), endotoxin and/or