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Effects of Anti-α1 Integrin Subunit Antibody on Anti-Thy-1 Glomerulonephritis

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SUMMARY: $\alpha1\beta1$ integrin is a potential collagen-binding extracellular matrix receptor that mediates collagen-dependent cell adhesion, proliferation, migration, and collagen matrix assembly and thereby may participate in the wound healing and pathologic scarring observed in some damaged organs. To clarify the role of $\alpha1\beta1$ integrin predominantly expressed on the mesangial cell (MC) surface in nephritic glomeruli, we investigated the involvement of MC-$\alpha1\beta1$ integrin in rat anti-Thy-1 glomerulonephritis (GN) by administering function-blocking monoclonal mouse anti-rat $\alpha1$ integrin subunit antibody (anti-$\alpha1$ Ab). Assay of collagen types I and IV mixed gel contraction, an in vitro model of pathologic collagen matrix remodeling, with function-blocking anti-$\alpha1$ Ab and anti-$\beta1$ Ab, revealed that collagen I and IV matrix reorganization is mediated by MC-$\alpha1\beta1$ integrin. In addition, conditioned medium from isolated Day 3 anti-Thy-1 nephritic glomeruli showed increased activity of MC-$\alpha1\beta1$ integrin-induced mixed collagen gel contraction as compared with that from isolated normal rat glomeruli. Treatment of Day 3 conditioned medium with anti–platelet-derived growth factor-BB antibody significantly inhibited conditioned media-induced gel contraction, whereas treatment with anti-transforming growth factor-β antibody did not have a significant effect. Rats that received anti-$\alpha1$ Ab from the left renal artery 3 days after anti-Thy-1 GN induction showed significant decreases of glomerular hypercellularity and mesangial matrix accumulation, including collagen I and IV in the left kidney, compared with those rats in which the left kidney received control mouse IgG1. These results suggest that MC-$\alpha1\beta1$ integrin is an important extracellular matrix receptor mediating mesangial remodeling characterized by MC proliferation and mesangial matrix reorganization in anti-Thy-1 GN. Platelet-derived growth factor-BB may be involved in early collagen matrix reorganization leading to pathologic mesangial remodeling in this GN model. (*Lab Invest 2002, 82:1219–1227*).

Abnormal mesangial remodeling is a key event leading to glomerular dysfunction and scarring (sclerosis) (Johnson, 1994; Prols et al, 1999). It is a prominent cell biologic feature of many progressive glomerular diseases and is characterized by extracellular matrix (ECM) accumulation and mesangial cell (MC) activation. As shown in human glomerulonephritis (GN) and experimental rat GN models, activated MCs acquire increased mitogenicity, migratory activity, de novo synthesis of α-smooth muscle actin (α-SM actin), and interstitial collagen types I and III (collagens I and III) (Hugo et al, 1996; Johnson, 1994). A major concept emerging from molecular cell biologic studies is that pathologic mesangial remodeling in progressive kidney diseases is caused by uncontrolled interactions between MCs, ECM, and growth factors (Kagami et al, 1993; Ruoslahti et al, 1994; Rupprecht et al, 1996).

$\beta1$ integrins, cell surface heterodimeric receptors consisting of a noncovalently associated common $\beta1$ subunit and several types of α subunits, mainly mediate interactions between cells and the ECM (Ruoslahti et al, 1994). This interactive process profoundly influences cellular metabolism and phenotype, therefore affecting cell growth, cell migration, cellular differentiation, and ECM assembly (Prols et al, 1999; Ruoslahti et al, 1994).

Among the $\beta1$ integrins, the $\alpha1\beta1$ integrin, a collagen and laminin receptor, has been shown to be involved in the collagen matrix reorganization observed in several damaged organs (Gotwals et al, 1996; Kagami et al, 1999; Racine-Samson et al, 1997). Increased expression of $\alpha1\beta1$ integrin by α-SM actin–expressing MCs accompanies abnormal ECM remodeling in rat and human GN (Kagami et al, 1993; Kuhara et al, 1997). Additionally, α-SM actin–expressing MCs respond via different mechanisms to transforming growth factor-β (TGF-β) and platelet-derived growth factor-BB (PDGF-BB) during stimulation of $\alpha1\beta1$ integrin-mediated collagen matrix reorganization when assayed in collagen I gel contraction assay, an established in vitro system of collagenous tissue re-

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modeling (Kagami et al, 1999). The TGF-β–stimulated gel contraction was accompanied by increased $\alpha1\beta1$ integrin expression, whereas PDGF-BB enhancement of gel contraction was found to be dependent on increased $\alpha1\beta1$ integrin-mediated MC migratory activity. Furthermore, Pozzi et al (1998) demonstrated using $\alpha1$-null fibroblasts that $\alpha1\beta1$ integrin is the sole collagen receptor delivering a specific collagen-induced signal to the Ras/ERK pathway leading to cell proliferation and migration. Recently, a substantial role for $\alpha1\beta1$ integrin in renal disease has been demonstrated in double-knockout mouse at both the collagen $\alpha3$(IV) gene (Alport mouse) and the $\alpha1$ integrin gene (Cosgrove et al, 2000). Extensive expansion of the mesangial matrix observed in Alport mice was attenuated in age-matched double-knockout mice. Altogether, the close relationship between activated MC-$\alpha1\beta1$ integrin action, ECM remodeling, and MC response to growth factors strongly supports a direct participation of $\alpha1\beta1$ integrin in pathologic mesangial remodeling (scar remodeling) in GN.

Here, we report results of a mixed collagen I and IV gel culture system, having a compositional similarity to an in vivo pathologic mesangial collagen matrix reorganization, and the administration of function-blocking mouse anti-$\alpha1$ Ab in rats with anti-Thy-1 GN. We demonstrate that reorganization of a collagen I and IV gel matrix is mediated by MC-$\alpha1\beta1$ integrin and is sensitive to PDGF-BB. In addition, we show that in vivo application of anti-$\alpha1$ Ab reduces MC-mediated pathologic mesangial matrix remodeling, suggesting that $\alpha1\beta1$ integrin is involved in the development of experimental GN.

Results

Immunohistochemical Analysis of Rat Anti-Thy-1 GN

The injection of anti-Thy-1 antibody into rats induces a rapid mesangiolysis within 1 to 2 days after disease induction, followed by MC migration, proliferation, and ECM remodeling, giving rise to the pathologic picture of mesangial proliferative GN (Johnson, 1994; Kagami et al, 1993). On Day 3, double immunostaining showed that most $\alpha1$ integrin-positive cells have both the expression of α-SM actin, a marker for activated MC, and of a moesin, a migratory cytoskeletal protein, from hilus to sparse peripheral MCs (Fig. 1, A and C). On Day 7, $\alpha1$ integrin-positive cells strongly expressed α-SM actin and moesin in a typical mesangial pattern of anti-Thy-1 GN (Fig. 1, B and D). Similarly, Hugo et al (1996) reported that mesangial expression of moesin paralleled the de novo expression of α-SM actin and was at a maximum on Day 7 of the same GN model. A previous study of ours showed that mesangial $\alpha1$ integrin staining is caused by exclusively MC-$\alpha1$ integrin staining and peaks at Day 7 of the disease (Kagami et al, 1993). Thus, activated MCs (+ α-SM actin) seem likely to show increased coexpression of $\alpha1$ integrin and a migratory protein, moesin, in anti-Thy-1 GN. In addition, glomerular deposition of pathologic collagen I was well colocalized with $\alpha1$ integrin-positive cells (Fig. 1, C and F). We chose Day 3 of GN as the treatment day because the injury phase of disease has passed and $\alpha1$ integrin-positive MCs have started to be activated and to organize pathologic collagen I within nephritic glomeruli (Floege et al, 1999).

Cell Biologic Analysis

The potential of MC-$\alpha1\beta1$ integrin for pathologic collagen matrix remodeling was investigated using a mixed collagen I and IV gel culture system (Fig. 2). Under standard conditions running MC-collagen gel incubation for 24 hours, MCs contracted the mixed collagen I and IV gels by almost 65%. However, function-blocking anti-$\beta1$ Ab completely inhibited the MC-induced gel contraction, whereas anti-$\alpha1$ Ab strongly inhibited but did not completely block it ($p < 0.01$; Fig. 2A). These results clearly indicate that $\alpha1\beta1$ integrin mediates collagen I and IV reorganization (Fig. 2A). Furthermore, isolated glomeruli from normal control and Day 3 GN rats were taken in cell culture to examine the mechanisms of how anti-$\alpha1$ Ab administered at Day 3 of GN affects nephritic glomeruli. After 24 hours of cultivation, the conditioned medium was taken and used in the mixed collagen I and IV gel culture system. Figure 2B shows that collagen gel contraction is significantly elevated in the presence of conditioned medium from Day 3 of nephritic glomeruli as compared with gel contraction from Day 0 of normal glomeruli ($p < 0.01$). When gels incubated with Day 3 conditioned media were supplemented with anti-$\beta1$ Ab, gel contraction by MCs was almost completely abolished at 24 hours. A weaker but comparable effect was observed by application of anti-$\alpha1$ Ab. These findings suggest that Day 3 conditioned medium-induced enhancement of gel contraction was $\alpha1\beta1$ integrin-mediated (Fig. 2B). To identify the nephritic glomeruli-secreted factors responsible for this observation, Day 3 conditioned medium was neutralized with antibodies against TGF-β or PDGF-BB, two representative growth factors known to play a role in the development of anti-Thy-1 GN. When the conditioned medium was pretreated with a neutralizing anti-TGF-β antibody before assay, there was no significant effect on the observed contractive activity of the cells in the same samples. However, when the same experiment was performed with medium pretreated with a neutralizing antibody to PDGF-BB, a significant decrease in conditioned medium induced-gel contraction occurred (Fig. 3). On the other hand, pretreatment of conditioned medium with control IgG had no effect on this assay. The effect of PDGF-BB was further demonstrated by undertaking a mixed collagen I and IV gel culture system with recombinant rat PDGF-BB in the presence or absence of a neutralizing anti-PDGF-BB antibody. As shown in Figure 4, recombinant PDGF-BB promoted the MC-dependent gel contraction. This effect was reversed in the presence of anti-PDGF-BB antibody.

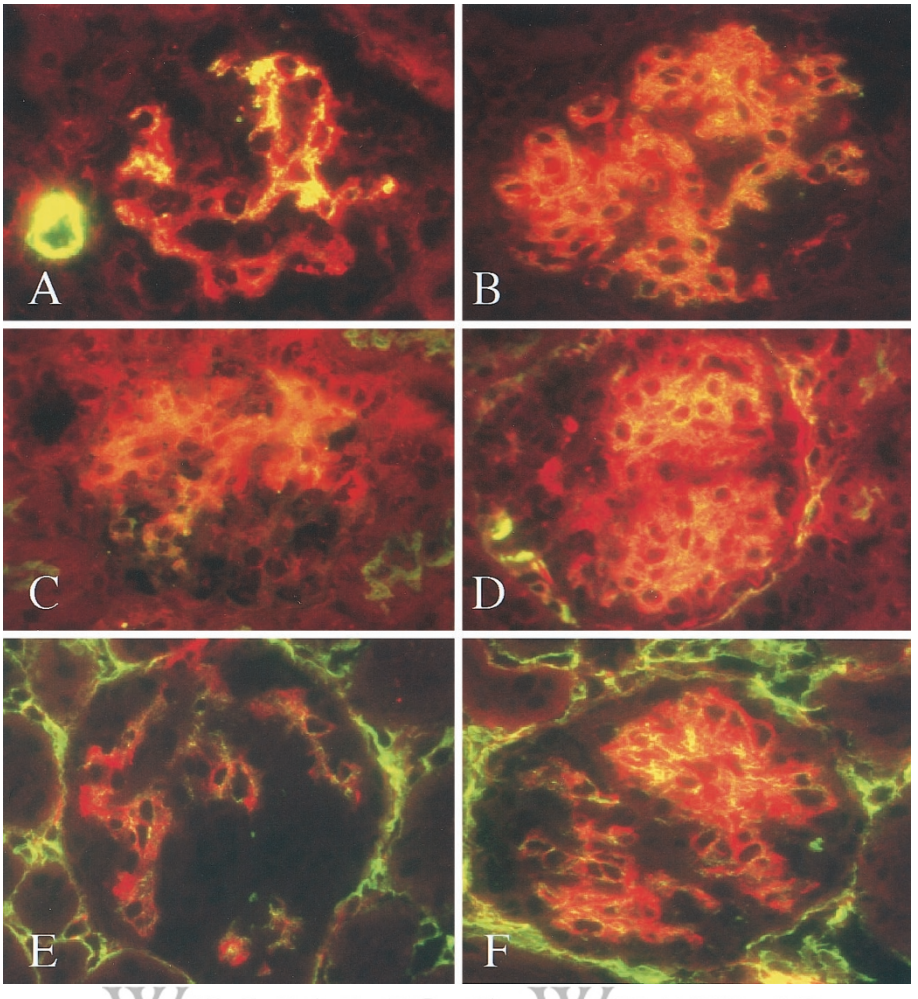

Figure 1.

Double immunostaining of α1 integrin subunit (TRITC in red) and α-smooth muscle actin (α-SM actin), moesin, and collagen type I (collagen I) (FITC in green) in glomeruli of anti-Thy-1 glomerulonephritis (GN) at Day 3 (A, C, and E) and Day 7 (B, D, and F). The kidney sections stained with rabbit anti-α1 integrin subunit antibody and TRITC-labeled anti-rabbit antibody were double stained with either mouse monoclonal anti-α-SM actin antibody (A and B), mouse monoclonal anti-moesin antibody (C and D), or goat anti-collagen I antibody (E and F), followed by an appropriate FITC-labeled anti-mouse antibody or FITC-labeled anti-goat IgG antibody. Yellow staining indicates areas of parallel expression of α1 integrin subunit and α-SM actin, moesin, or collagen I. Note the colocalization of collagen I deposition with α1 integrin-positive cells in the nephritic glomeruli (E and F) (Original magnification, ×200).

Effect of In Vivo Administration of Monoclonal Anti-α1 Integrin Antibody on Anti-Thy-1 GN

To examine the in vivo function of α1 integrin in anti-Thy-1 GN, function-blocking anti-α1 Ab was injected into the left kidneys of anti-Thy-1 GN rats on Day 3. Four days after injection (Day 7 of anti-Thy-1 GN), kidneys of rats were isolated and subjected to histochemistry and immunohistochemistry. A representative periodic acid-Schiff (PAS) stain on Day 7 is shown in Figure 5; the semiquantitation of the matrix score and cell proliferation is shown in Figure 6. On Day 7 of anti-Thy-1 GN, marked MC proliferation and ECM accumulation was observed in rat left kidneys treated with mouse IgG1 (Fig. 5B). Of note, administration of anti-α1 Ab into the left kidneys showed reduced mesangial proliferative changes at Day 7 (Fig. 5C). There was a significant decrease of glomerular hypercellularity and matrix score in the left kidney of rats treated with anti-α1 Ab compared with those rats receiving mouse IgG1 (59 ± 5.2 versus 82 ± 11 cells/glomerulus; 1.6 ± 0.3 versus 3.0 ± 0.6, $p < 0.01$, respectively) (Fig. 6, A and B). Immunostaining of kidney sections shows marked increases in the expression of α-SM actin and collagens I and IV, which were predominantly detected in mesangial lesions of mouse IgG1-treated rats (Fig. 7). In contrast, the degree of glomerular expression of α-SM actin and collagen I and IV decreased significantly in anti-α1 Ab–treated rats ($p < 0.01$ for each protein). The number of cells positive for the monocyte/macrophage marker ED-1 was examined in each group on Day 7. The mouse IgG1-treated and anti-α1 Ab–treated rats revealed 2 ± 0.3 and 2 ± 0.2 ED-1–positive cells per Day 7 glomerular section, respectively, indicating that administration of anti-α1 Ab into Day 3 anti-Thy-1 GN rats had no significant effect on monocyte/macrophage infiltration into Day 7 glomeruli (normal rat; 1 ± 0.4). The treatment of anti-Thy-1 rats with anti-α1 Ab reduced the mean proteinuria on Day 7, but this failed to reach statistical significance when

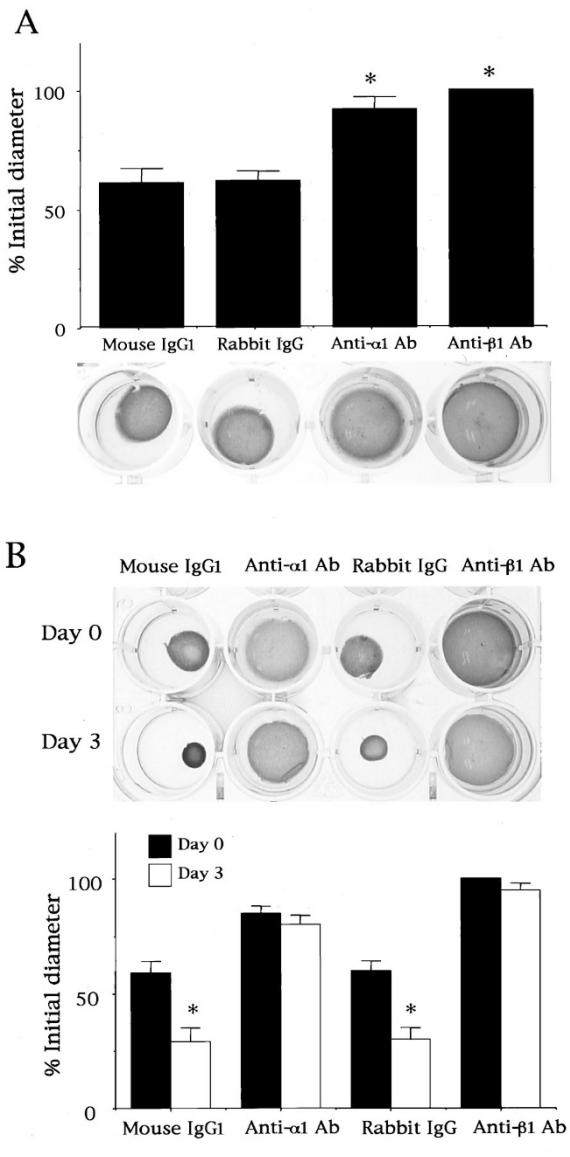

Figure 2.

Effect of monoclonal anti-rat α1 integrin subunit antibody (anti-α1 Ab) or polyclonal anti-β1 integrin antibody (anti-β1 Ab) on mesangial cell (MC)-mediated contraction of collagen I and IV gels in the presence or absence of conditioned medium. A, Collagen gels were prepared using a collagen I and IV mixture. MCs suspended in RPMI medium were added in neutralized collagen solution and poured into 24-well plates with either anti-α1 Ab (50 μg/ml), anti-β1 Ab (100 μg/ml), or an appropriate control (rabbit IgG or mouse IgG1). The degree of mixed collagen gel contraction by MCs in the presence of anti-β1 Ab or anti-α1 Ab was compared with that in the presence of an appropriate control (* $p < 0.01$ versus control IgG). B, MCs were added to a neutralized mixed collagen solution and poured into 24-well plates with conditioned medium obtained from either isolated Day 0 (normal) or Day 3 (nephritic) glomeruli in 24-hour culture. The degree of gel contraction induced by MCs coincubated with Day 0 conditioned medium (Day 0) was compared with that of MCs coincubated with Day 3 conditioned medium (Day 3) (* $p <$ 0.01; Day 0 versus Day 3). Striking inhibitory effects of gel contraction are observed in combinations of culture media with either anti-α1 Ab or anti-β1 Ab.

compared with rats treated with mouse IgG1 (60 $\pm$ 10 versus 49 $\pm$ 9 mg/24 hours). These results suggested that anti-α1 Ab suppressed MC proliferation and collagen accumulation leading to mesangial ECM expansion in anti-Thy-1 GN rats.

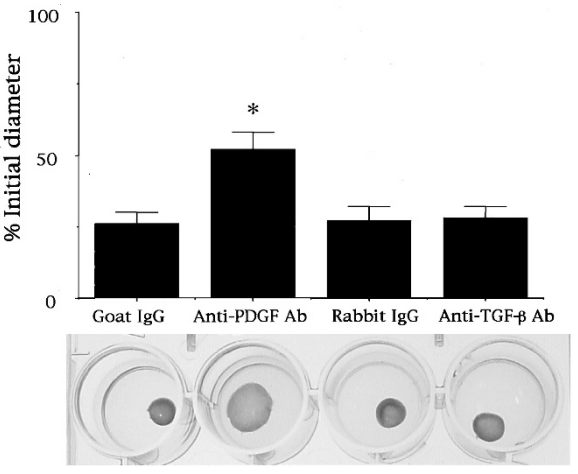

Figure 3.

Effects of neutralizing anti-platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β (TGF-β) antibodies on MC-mediated contraction of mixed collagen I and IV gels prepared with Day 3 conditioned medium. Conditioned medium from Day 3 anti-Thy-1 nephritic glomeruli was mixed with either goat anti-PDGF-BB antibody (anti-PDGF Ab; 20 μg/ml), rabbit anti-TGF-β antibody (anti-TGF-β Ab; 20 μg/ml), or an appropriate control IgG (20 μg/ml) and incubated at 4° C overnight. MCs were added into a mixed collagen I and IV solution with treated conditioned medium and followed by gel contraction assay. The degree of gel contraction induced by Day 3 conditioned medium treated with either anti-PDGF Ab or anti-TGF-β Ab was compared with those of the counterpart treated with control goat IgG or rabbit IgG (* $p < 0.01$ versus control goat IgG).

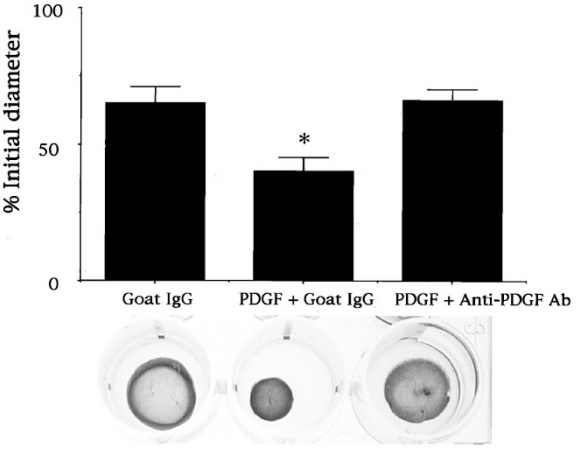

Figure 4.

Effect of rat PDGF-BB on MC-mediated contraction of collagen I and IV gels. MCs were added into a neutralized mixed collagen solution and poured into 24-well plates with either goat IgG (20 μg/ml), rat recombinant PDGF-BB (PDGF; 50 μg/ml) and goat IgG (20 μg/ml) or PDGF (50 μg/ml) and goat anti-PDGF-BB antibody (anti-PDGF Ab; 20 μg/ml). The degree of MCs-induced gel contraction treated with PDGF and goat IgG was compared with those of MCs-induced gel contraction treated with either goat IgG or PDGF and anti-PDGF Ab (* $p < 0.01$ versus goat IgG or PDGF + anti-PDGF Ab).

Discussion

Cell proliferation, migration, and ECM remodeling are basic cell biologic responses of MCs to the various types of glomerular injuries and contribute to the mesangial structural and functional alterations seen in many types of rat and human GN (Barnes et al, 1994; Hugo et al, 1996; Johnson, 1994). In particular, per-

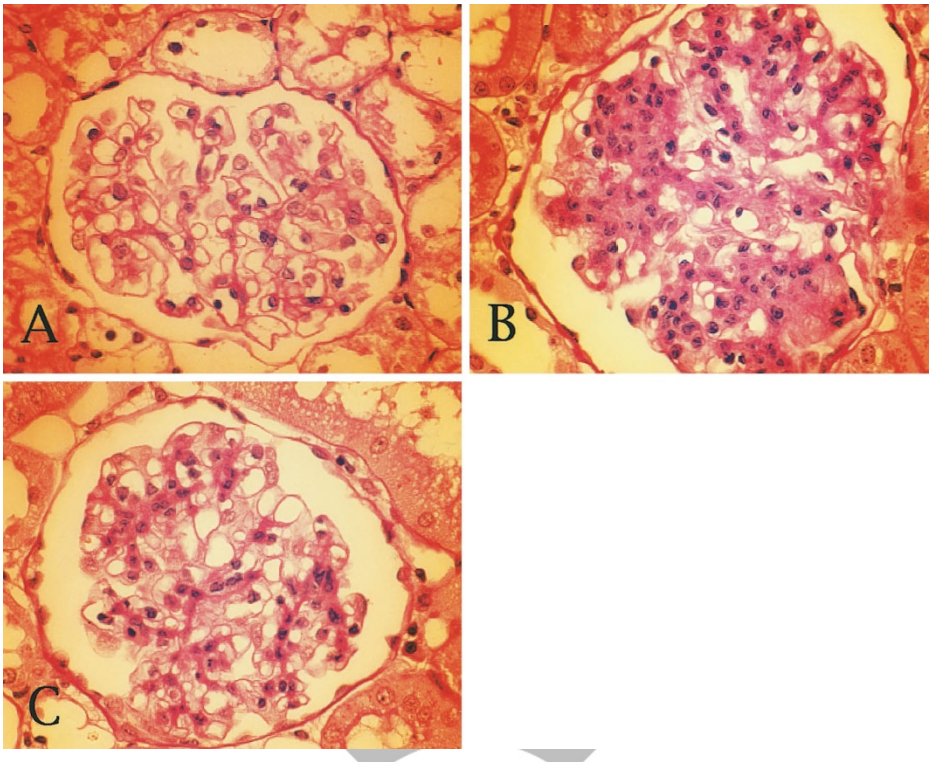

Figure 5.
Effect of mouse anti-α1 Ab on rats with anti-Thy-1 GN. Rats were injected with either mouse IgG1 or anti-α1 Ab in the left renal artery 3 days after induction of anti-Thy-1 GN. Micrographs show representative results of PAS staining of glomeruli from normal rat (A) and Day 7 anti-Thy-1 GN rats treated with either mouse IgG1 (B) or anti-α1 Ab (C) (Original magnification, ×200).

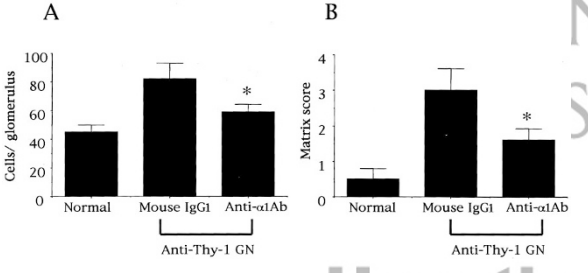

Figure 6.
Quantitation of the effect of mouse anti-α1 Ab on anti-Thy-1 GN on Day 7 (A and B). The degree of glomerular damage of rats treated with either mouse IgG1 or anti-α1 Ab was evaluated by total glomerular cell counts (A) or matrix score (B). Data are shown as mean ± SD (* $p < 0.01$ versus control mouse IgG1).

sistent abnormal mesangial remodeling of ECM components such as collagens I and IV is a hallmark leading to glomerulosclerosis (Johnson, 1994; Kagami et al, 1993; Peten et al, 1992). Although it has been demonstrated that ECM remodeling is an integrin-mediated event (Giancotti and Ruoslahti, 1990; Gotwals et al, 1996; Kagami et al, 1999), little is known about the pathophysiologic role of a major collagen-binding receptor, α1β1 integrin, expressed on glomerular MCs, in vivo in normal and diseased states (Cosgrove et al, 2000).

The present study provides evidence that α1β1 integrin is a potent ECM receptor mediating MC proliferation and ECM remodeling after acute glomerular injury in rat anti-Thy-1 GN. Immunohistochemical analyses showed that α1β1 integrin-positive MCs have characteristics of a contractile/migratory phenotype in anti-Thy-1 nephritic glomeruli. Administration of a function-blocking anti-α1 integrin antibody on Day 3 after induction of nephritis caused a significant reduction in glomerular changes, as indicated by histologic evaluations such as hypercellularity and accumulation of ECM, including collagens I and IV. Further support for the reduction of MC proliferation is provided by the observation that glomerular de novo expression of α-SM actin was significantly inhibited by anti-α1 Ab. Recently, α1β1 integrin has been demonstrated to be the sole collagen receptor mediating collagen-dependent cell proliferation in vivo (Pozzi et al, 1998). We reported that rat MCs predominantly expressed α1β1 integrin in vivo and in vitro and also used this receptor for adhesion to collagens I and IV in vitro (Kagami et al, 1993, 1999). Therefore, anti-α1 Ab seems to affect MC proliferation by inhibiting the mediation of α1β1 integrin required for cell proliferation in anti-Thy-1 GN.

With respect to ECM accumulation in GN, we have reported by using collagen I gel contraction assay that fibrogenic growth factors such as PDGF-BB and TGF-β stimulate MC- α1β1 integrin–mediated pathologic collagen I reorganization and thereby may contribute to the development of glomerular sclerosis (Kagami et al, 1999). Additionally, we have shown here, using a mixed collagen gel assay composed of collagens I and IV with a similarity to damaged mes-

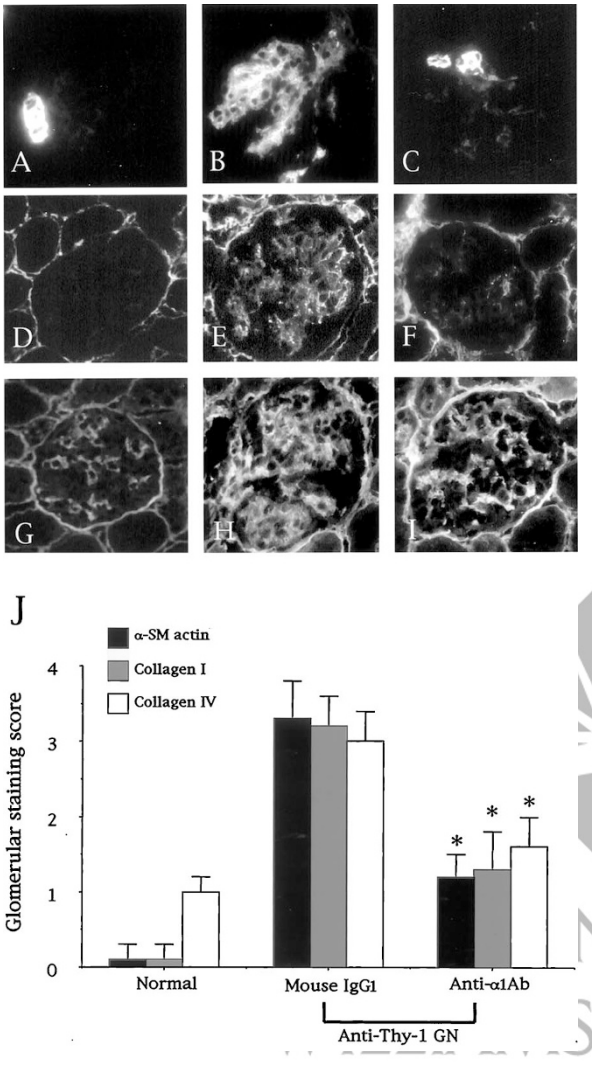

Figure 7.

Renal expression of α-SM actin and collagens I and IV in normal and Day 7 anti-Thy-1 GN rats. Immunofluorescence for α-SM actin (A to C), collagen I (D to F), and collagen IV (G to I) in kidney sections from normal rats (A, D, and G) and Day 7 anti- Thy-1 GN rats treated with either mouse IgG1 (B, E, and H) or anti-α1 Ab (C, F, and I) (Original magnification, $\times$200). J, Semiquantitative assessment of immunofluorescence staining of α-SM actin and collagens I and IV in normal rats and Day 7 anti-Thy-1 GN rats treated with either mouse IgG1 or anti-α1 Ab. Data are shown as mean $\pm$ SD (*$p < 0.01$ versus control mouse IgG1).

angial collagen matrix organization in vivo, that culture medium from isolated Day 3 anti-Thy-1 nephritic glomeruli contained stimulatory factors, including PDGF-BB, to enhance MC-$\alpha1\beta1$ integrin–mediated collagen I and IV matrix reorganization. Newly elaborated collagen matrices in damaged glomeruli provide a foundation for subsequent matrix assembly, leading to excess mesangial matrix accumulation because collagens I and IV have many binding domains for other ECM molecules such as fibronectin (Charonis et al, 1985; Shimizu et al, 1997). This may explain the mechanism by which administration of anti-α1 Ab into Day 3 nephritic rats reduced the pathologic mesangial matrix accumulation subsequently observed in Day 7 nephritic rats.

It is well established that in the anti-Thy-1 nephritis model, the acute phase characterized by MC proliferation and migration is PDGF dependent, whereas the later phase characterized by glomerular ECM accumulation is TGF-β dependent (Border et al, 1990; Floege et al, 1999). We previously reported that PDGF stimulates collagen matrix remodeling by cultured MCs via enhanced $\alpha1\beta1$ integrin–mediated MC migratory activity. The results of the present study show that α1 integrin-positive MCs strongly expressed the migratory protein, moesin, in anti-Thy-1 GN. Taken together, these results show that the enhanced PDGF activity in the acute phase of this GN may also contribute to increased collagen I and IV matrix reorganization through enhancement of $\alpha1\beta1$ integrin-dependent MC migratory activity. Furthermore, a recent report has demonstrated that the extensive expansion of the mesangial matrix observed in Alport mice at 7 weeks of age was attenuated in age-matched double-knockout mice at both the collagen α3(IV) gene (Alport mouse) and the α1 integrin gene, indicating the involvement of $\alpha1\beta1$ integrin in progressive mesangial ECM accumulation of renal diseases (Cosgrove et al, 2000). However, because administration of the anti-α1 Ab did not completely block the pathologic changes in anti-Thy-1 GN, it is possible that there might be other factors required for the development of severe mesangial proliferative GN.

Several studies have reported that $\alpha1\beta1$ integrin on activated mesenchymal cells (myofibroblasts) play a pathophysiologic role in wound repair and/or fibrotic process in injured organs. Further data by Gotwals et al (1996) received from use of a rat vascular injury model in combination with cell culture experiments indicated that $\alpha1\beta1$ integrin is a critical collagen receptor involved in vascular wound healing by smooth muscle cells. Racine-Samson et al (1997) suggested (using the activated stellate cells isolated from a rat model of liver injury) that $\alpha1\beta1$ integrin contributes to the development of liver fibrosis. Interestingly, it has been shown that blocking of α1 integrin mitigates the inflammatory response in murine models of contact sensitivity, delayed-type hypersensitivity, and arthritis (de Fougerolles et al, 2000). Additionally, the study on the α1 integrin deletion in Alport mice demonstrated that α1 integrin is required for the accumulation of monocytes in damaged renal interstitium, indicating that $\alpha1\beta1$ integrin on macrophage cell surfaces is involved in the chronic inflammatory process in renal interstitium (Sampson et al, 2001). Our single administration of anti-α1 Ab into an acute model of anti-Thy-1 GN did not reveal a significant role for $\alpha1\beta1$ integrin on macrophages in nephritic glomeruli, and further investigation is needed to clarify the role of $\alpha1\beta1$ integrin on macrophages in renal fibrosis using rat models of chronic GN with a progressive nature. Taken together, $\alpha1\beta1$ integrin seems likely to be a common target molecule for regulating the fibrotic and inflammatory tissue reaction against injury.

In conclusion, our studies demonstrate that $\alpha1\beta1$ integrin mediates MC proliferation and ECM accumu-

lation in anti-Thy-1 GN. Glomerular PDGF-BB activity may contribute to the pathologic mesangial matrix remodeling through the enhancement of α1β1 integrin–dependent MC migratory activity. Considering that α1β1 integrin plays a role in activated mesenchymal cells-induced tissue remodeling in many organs, the modulation of α1β1 integrin expression by activated MCs in nephritic glomeruli may provide a new therapeutic approach for regulating abnormal mesangial remodeling leading to glomerulosclerosis.

Materials and Methods

ECM Proteins, Growth Factors, and Antibodies

Rat tail collagen I was obtained from Collaborative Biomedical Products (Bedford, Massachusetts). Bovine collagen IV was purified and characterized as previously described (Hirose et al, 1999). The purity of these matrix proteins was verified by SDS-PAGE. Goat neutralizing anti-PDGF-BB and rabbit neutralizing TGF-β antibodies were purchased from R&D Systems (Minneapolis, Minnesota), as was recombinant rat PDGF-BB. Mouse monoclonal anti-moesin antibody was a kind gift from Dr. Shoichiro Tsukita (University of Kyoto, Japan) and was used to detect migratory protein in MCs (Doi et al, 1999). Mouse monoclonal anti-α-SM actin antibody (1A4) was purchased from Sigma Chemical Company (St. Louis, Missouri). Goat anti-collagen I and IV antibodies were obtained from Southern Biotechnology Associates (Birmingham, Alabama). Mouse monoclonal anti-rat monocyte/macrophage antibody (anti-ED-1) was purchased from Serotec (Oxford, United Kingdom). A function-blocking monoclonal mouse anti-α1 Ab (IgG1) and polyclonal rabbit anti-rat β1 integrin subunit IgG (anti-β1 Ab) were produced and characterized as previously described (Löster et al, 1994). Each antibody was directed to the extracellular domain of the β1 or α1 integrin subunit, respectively, and could disrupt MC adhesion to ECM proteins with ligand specificity. Polyclonal rabbit anti-rat α1 integrin subunit antibody was produced as previously described (Löster et al, 1994). Mouse myeloma IgG1 (Zymed Laboratories Inc., San Francisco, California), goat IgG, and rabbit IgG (R&D Systems) were used as control antibodies.

Induction of Anti-Thy-1 GN

Anti-Thy-1 mesangial proliferative GN was induced in 8-week-old male Sprague-Dawley rats (SLC, Shizuoka, Japan) by intravenous injection of sheep anti-rat Thy-1 serum (0.25 ml/100 gm body weight) via the tail vein (Kagami et al, 1994). Nephritic rats without any treatment ($n = 10$) were killed to examine the characteristics of α1 integrin-positive cells on Days 3 and 7 of GN. Treatment consisted of left renal perfusion of mouse IgG1 (2 mg) or anti-α1 Ab 33.4 (2 mg) into nephritic rats ($n = 10$ per each treatment) at Day 3 after induction of anti-Thy-1 GN. These rats were killed for histologic examination of kidney tissue on Day 7 of GN. Animals were housed in metabolic cages from Days 0 to 1, from Days 2 to 3, and from Days 6 to 7, and total urine output was collected. Twenty-four hour urinary protein content was measured using a BCA protein assay kit (Pierce, Rockford, Illinois) and bovine serum albumin as a standard. Normal ranges of proteinuria and renal histologic parameters were established in 10 nonmanipulated Sprague-Dawley rats of similar ages.

Histology and Immunohistochemistry

Kidney tissue from each rat was processed and examined by light immunofluorescence as previously described (Kagami et al, 1994). Frozen sections (3 μm) were fixed in acetone. They were then incubated with the rabbit anti-α1 integrin subunit antibody for 1 hour and subsequently with tetramethylrhodamine isothiocyanate (TRITC)-coupled donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). In double-staining experiments, the sections were further incubated with either mouse monoclonal anti-α-SM actin antibody, mouse monoclonal anti-moesin antibody, goat anti-human collagen I antibody or goat anti-human collagen IV antibody, followed by an appropriate FITC-coupled donkey anti-mouse antibody or FITC-coupled donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, Inc.). Negative controls included omission of either of the primary antibodies in which no double staining was noted.

For evaluation of the level of glomerular staining with each anti-collagen I antibody, anti-collagen IV antibody, and anti-α-SM actin antibody, semiquantitative analysis was performed as follows: 0, diffuse, very weak or absent mesangial staining; 1+, diffuse, weak mesangial staining with 1% to 25% of focally increased mesangial staining; 2+, 25% to 50% of glomerular tuft demonstrating strong mesangial staining; 3+, 50% to 75% of glomerular tuft demonstrating strong mesangial staining; and 4+, 75% or more of glomerular tuft stained strongly. For each kidney section, 30 glomeruli were selected at random and were evaluated by a blinded observer; the mean value per section was calculated. For the kidney sections stained by anti-ED-1, 30 glomeruli were selected randomly and the number of positively stained cells in each glomerulus was counted.

For semiquantitative evaluation of mesangial matrix accumulation and glomerular hypercellularity, all 3-μm PAS-stained sections were coded and read by a blinded observer. At least 30 glomeruli were selected at random, cell nuclei were counted, and the degree of glomerular matrix expansion was determined using a published method (Okuda et al, 1990). The percentage of each glomerulus occupied by mesangial matrix was estimated and assigned a code as follows: 1 = 0% to 25%, 2 = 25% to 50%, 3 = 50% to 75%, and 4 = 75% to 100%.

Cell Culture

Rat MCs were obtained from intact glomeruli of 6-week-old Sprague-Dawley rats and characterized

according to published methods (Kagami et al, 1994). MCs were used between passages 5 and 9 and were maintained in RPMI 1640 medium (Sigma Chemical Company) supplemented with 18% fetal bovine serum (Gibco BRL, Grand Island, New York), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 U/ml insulin, 25 mM HEPES buffer at 37° C in a 5% CO_2 incubator. All MC culture experiments were performed after a 2-day incubation in serum-free RPMI 1640 to reach MC quiescence (Kagami et al, 1994). Rat glomeruli obtained from normal rats ($n = 5$) and Day 3 anti-Thy-1 GN–induced rats ($n = 5$) by the graded sieving technique were suspended at 10^4 glomeruli/ml in serum-free RPMI 1640 medium in 6-well multiplates (Corning Glassworks, Corning, New York). After 24 hours of incubation at 37° C in a CO_2 incubator, the respective conditioned media were harvested and centrifuged to remove particulates and cells. These conditioned media were then stored at −20° C.

Mixed Collagen I and IV Gel Contraction Assay

Mixed collagen gel assays were performed to examine the ability of MCs to reorganize and contract three-dimensional mixed collagen I and IV gels. The $\alpha1\beta1$ integrin expressed on MCs is a major receptor for collagens I and IV as previously reported (Kagami et al, 1999). Concomitant expression of a normal constituent, collagen IV, and an abnormal constituent, interstitial collagen I, was often observed in damaged mesangium in various types of GN. Therefore, this assay system offers us a similar situation to in vivo pathologic collagen matrix remodeling by MCs. Quiescent MCs were harvested and suspended in the absence of anti-$\beta1$ Ab or anti-$\alpha1$ Ab 33.4 at a concentration of 5×10^5 cells/ml in 0.5 ml of 1.25 × RPMI 1640 for 30 minutes at 20° C. The cell suspension was mixed on ice with 0.5 ml of collagen I and IV solution (72.2% 1.30 × RPMI 1640, 10% rat tail collagen I [3.5 mg/ml], 17.5% bovine collagen IV [2 mg/ml], 0.3% 2N NaOH). Collagen/cell suspensions (500 μl each) were incubated in 24-well plates (Costar, Cambridge, Massachusetts) at 37° C to polymerize the collagen. The diameter of the hydrated gels was measured by use of an inverted microscope at 24 hours. To examine the effects of conditioned media on mixed gel contraction, 50 μl of conditioned medium obtained from either normal rat or nephritic glomeruli was added to the collagen/cell suspension before gel polymerization. Furthermore, the growth factors responsible for conditioned media–induced gel contraction were investigated by using overnight preincubated conditioned media with either neutralizing anti-PDGF-BB antibody (20 μg/ml), neutralizing anti-TGF-β antibody (20 μg/ml), or an appropriate control antibody (20 μg/ml) at 4° C.

Statistical Analysis

Results are presented as mean ± SD. A value of $p < 0.05$ was used to determine statistical significance (Student's t test). Triplicate wells were analyzed for each experiment, and each experiment was performed independently at a minimum of three times.

References

Barnes JL, Hevey KA, Hastings RR, and Bocanegra RA (1994). Mesangial cell migration precedes proliferation in habu snake venom-induced glomerular injury. Lab Invest 70:460–467.

Border WA, Okuda S, Languino LR, Sporn MB, and Ruoslahti E (1990). Suppression of experimental glomerulonephritis by antiserum against transforming growth factor-$\beta1$. Nature 346:371–374.

Charonis AS, Tsilibary EC, Yurchenco PD, and Furthmayr H (1985). Binding of laminin to type IV collagen: A morphological study. J Cell Biol 100:1848–1853.

Cosgrove D, Rodgers K, Meehan D, Miller C, Bovard K, Gilroy A, Gardner H, Kotelianski V, Gotwals P, Amatucci A, and Kalluri R (2000). Integrin $\alpha1\beta1$ and transforming growth factor-$\beta1$ play distinct roles in alport glomerular pathogenesis and serve as dual targets for metabolic therapy. Am J Pathol 157:1649–1659.

de Fougerolles AR, Sprague AG, Nickerson-Nutter CL, Chi-Rosso G, Rennert PD, Gardner H, Gotwals PJ, Lobb RR, and Koteliansky VE (2000). Regulation of inflammation by collagen-binding integrins $\alpha1\beta1$ and $\alpha2\beta1$ in models of hypersensitivity and arthritis. J Clin Invest 105:721–729.

Doi Y, Itoh M, Yonemura S, Ishihara S, Takano H, Noda T, and Tsukita S (1999). Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout. J Biol Chem 274:2315–2321.

Floege J, Ostendorf T, Janssen U, Burg M, Radeke HH, Vargeese C, Gill SC, Green LS, and Janjic N (1999). Novel approach to specific growth factor inhibition in vivo: Antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. Am J Pathol 154:169–179.

Giancotti FG and Ruoslahti E (1990). Elevated levels of the $\alpha5\beta1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell 60:849–859.

Gotwals PJ, Chi-Rosso G, Lindner V, Yang J, Ling L, Fawell SE, and Koteliansky VE (1996). The $\alpha1\beta1$ integrin is expressed during neointima formation in rat arteries and mediates collagen matrix reorganization. J Clin Invest 97:2469–2477.

Hirose M, Kosugi H, Nakazato K, and Hayashi T (1999). Restoration to a quiescent and contractile phenotype from a proliferative phenotype of myofibroblast-like human aortic smooth muscle cells by culture on type IV collagen gels. J Biochem 125:991–1000.

Hugo C, Hugo C, Pichler R, Gordon K, Schmidt R, Amieva M, Couser WG, Furthmayr H, and Johnson RJ (1996). The cytoskeletal linking proteins, moesin and radixin, are upregulated by platelet-derived growth factor, but not basic fibroblast growth factor in experimental mesangial proliferative glomerulonephritis. J Clin Invest 97:2499–2508.

Johnson RJ (1994). The glomerular response to injury: Progression or resolution? Kidney Int 45:1769–1782.

Kagami S, Border WA, Ruoslahti E, and Noble NA (1993). Coordinated expression of $\beta1$ integrins and transforming growth factor-β-induced matrix proteins in glomerulonephritis. Lab Invest 69:68–76.

Kagami S, Border WA, Miller DE, and Noble NA (1994). Angiotensin II stimulates extracellular matrix synthesis through induction of transforming growth factor-β expression in rat glomerular mesangial cells. J Clin Invest 93:2431–2437.

Kagami S, Kondo S, Löster K, Reutter W, Kuhara T, Yasutomo K, and Kuroda Y (1999). α1β1-integrin-mediated collagen matrix remodeling by rat mesangial cells is differentially regulated by transforming growth factor-β and platelet-derived growth factor-BB. J Am Soc Nephrol 10:779–789.

Kuhara T, Kagami S, and Kuroda Y (1997). Expression of β1-integrins on activated mesangial cells in human glomerulonephritis. J Am Soc Nephrol 8:1679–1687.

Löster K, Voigt S, Heidrich C, Hofmann W, and Reutter W (1994). Cell-collagen adhesion is inhibited by monoclonal antibody 33.4 against the rat α_1-integrin subunit. Exp Cell Res 212:155–160.

Okuda S, Languino LR, Ruoslahti E, and Border WA (1990). Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis: Possible role in expansion of the mesangial extracellular matrix. J Clin Invest 86:453–462.

Peten EP, Striker LJ, Carome MA, Elliott SJ, Yang CW, and Striker GE (1992). The contribution of increased collagen synthesis to human glomerulosclerosis: A quantitative analysis of α2IV collagen mRNA expression by competitive polymerase chain reaction. J Exp Med 176:1571–1576.

Pozzi A, Wary KK, Giancotti FG, and Gardner HA (1998). Integrin α1β1 mediates a unique collagen-dependent proliferation pathway in vivo. J Cell Biol 142:587–594.

Prols F, Hartner A, Schöcklmann HO, and Sterzel RB (1999). Mesangial cells and their adhesive properties. Exp Nephrol 7:137–146.

Racine-Samson L, Rockey DC, and Bissell DM (1997). The role of α1β1 integrin in wound contraction. J Biol Chem 272:30911–30917.

Ruoslahti E, Noble NA, Kagami S, and Border WA (1994). Integrins. Kidney Int 45(Suppl 44):S17–S22.

Rupprecht HD, Schocklmann HO, and Sterzel RB (1996). Cell-matrix interactions in the glomerular mesangium. Kidney Int 49:1575–1582.

Sampson NS, Ryan ST, Enke DA, Cosgrove D, Koteliansky V, and Gotwals P (2001). Global gene expression analysis reveals a role for the α1 integrin in renal pathogenesis. J Biol Chem 276:34182–34188.

Shimizu M, Minakuchi K, Moon M, and Koga J (1997). Difference in interaction of fibronectin with type I collagen and type IV collagen. Biochim Biophys Acta 1339:53–61.