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Short Communication

Cyclic Stretching of Mesangial Cells Up-Regulates Intercellular Adhesion Molecule-1 and Leukocyte Adherence

A Possible New Mechanism for Glomerulosclerosis

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Intraglomerular hypertension is a primary causal factor in the progressive glomerulosclerosis that characterizes diabetic nephropathy or severe renal ablation. However, inflammation of the glomerular mesangium also participates in at least the early phase of these diseases. In glomerulonephritis, where inflammation is thought to be the predominant causal factor, intraglomerular hypertension is also often present. Mesangial cells (MCs) are critical in orchestrating key functions of the glomerulus including extracellular matrix metabolism, cytokine production, and interaction with leukocytes. Because MCs are subject to increased stretching when intraglomerular hypertension is present, and in glomerulonephritis MC/leukocyte interactions seem to be mediated primarily via the up-regulation of intercellular adhesion molecule-1 (ICAM-1), we examine the possibility that cyclic stretching is a stimulus for increased MC ICAM-1 activity. We demonstrate that the normal low levels of MC ICAM-1 mRNA and protein are dramatically up-regulated by even short intervals of cyclic stretch. This effect is dose- and time-dependent, and requires little amplitude and a brief period of elongation for significant induction. Stretch-induced MC ICAM-1 also leads to a marked elevation in phagocytic leukocyte adherence. This stimulated adherence is equal or greater than that induced by the inflammatory cytokine tumor necrosis factor-α, whereas an additive effect occurs when both are applied in combination. Our results indicate that stretch-induced ICAM-1 may provide a direct link between hypertension and inflammation in the progression of injury and glomerulosclerosis in diabetes, renal ablation, and other forms of glomerulonephritis. *(Am J Pathol 2001, 158:11–17)*

Progressive glomerular disease resulting from a variety of pathological events is characterized by mesangial cell (MC) injury[1] and/or the increased deposition of extracellular matrix (ECM).[2] One well-established cause is glomerular hypertension.[3] The mechanisms whereby glomerular hypertension leads to MC injury and/or ECM overaccumulation are only beginning to be identified. However, it is known that the expansion of glomerular structures during increased intracapillary pressure leads to enhanced MC mechanical strain.[4] MCs in culture respond to such strain with increased production and binding of the prosclerotic cytokines, transforming growth factor-β and connective tissue growth factor,[4–6] and the accumulation of ECM.[7,8]

In addition to the glomerular hemodynamic alteration, leukocyte infiltration is a factor that plays an important role in the initial kidney damage and consequent sclerosis that is associated with various forms of glomerular injury. Phagocytic leukocytes (neutrophils, monocytes, and macrophages) infiltrate glomeruli early in acute glomerulonephritis and play a key role in its pathogenesis.[9] It is now clear that an influx of phagocytic leukocytes into the glomerulus is also a response to renal ablation[10–12]

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and to experimental diabetes.[13] The process of glomerular inflammation requires migration in response to a chemokine gradient, diapedesis through an endothelial barrier, and interaction with resident renal cells via adhesion molecules.[14] Intercellular adhesion molecule-1 (ICAM-1), a transmembrane glycoprotein and member of the immunoglobulin superfamily, seems to be the most important adhesion molecule in this process.[15] It is expressed on many hematopoietic and nonhematopoietic cells and its ligands, or counterreceptors, on leukocytes including the β integrins, CD11a/CD18 and CD11b/CD18.[16] Although the glomerular expression of ICAM is normally low or absent, it is up-regulated in the mesangium in many forms of glomerulonephritis including focal segmental glomerulosclerosis, early rapidly progressive glomerulonephritis, mesangioproliferative glomerulonephritis, IgA nephropathy, and lupus nephritis, as shown in both human[17,18] and animal models.[15,19,20] Recent studies in rat glomeruli have also shown an up-regulation of ICAM-1 after streptozotocin-induced diabetes and 5/6 nephrectomy.[21,22] The mechanisms for induction of ICAM in the glomerulus are not clear. However, the low constitutive ICAM-1 expressed on MCs in culture is increased after exposure to tumor necrosis factor (TNF) or interferon (IFN)-γ, or interleukin (IL)-1, suggesting that certain inflammatory cytokines released from infiltrating leukocytes are key regulatory factors in the glomerulus.[14,23]

A role for hypertensive force in the expression of ICAM has not been reported. However, it has been shown that after renal ablation in rats, treatments with the immunosuppressive agent mycophenolate mofetil, that blocks ICAM-1 up-regulation, correspondingly prevents proteinuria without an effect on arterial pressure.[24] Also, sheer stress, another form of mechanical force, has been reported to increase ICAM expression on endothelial[25] and epithelial[26] cells. We hypothesized that cyclic strain resulting from glomerular hypertension would up-regulate the production and localization of MC ICAM-1, thus allowing increased interaction with leukocytes. This physical interaction would then result in MC activation and/or injury with subsequent glomerular damage occurring via inflammatory processes and acceleration of ECM accumulation.

Materials and Methods

Cells

The MCs, previously characterized by us, were a cloned line derived from outgrowths of Fischer rat glomeruli.[7] On serial passage, they continue to express key markers of MCs,[7] including Thy-1. They demonstrate the same high sensitivity to phorbol-stimulated neutrophil adhesion and lysis, as do early passage MCs indicating continued and like expression of essential cell surface receptors.[16] The medium used was RPMI 1640 with penicillin and streptomycin and 8 mmol/L glucose and (unless otherwise noted) 20% Nu-Serum from Collaborative Research, (Bedford, MA). Human peripheral blood leukocytes were obtained from healthy adult volunteers and isolated as previously described.[27] The cells obtained were ~98% polymorphonuclear granulocytes and were suspended in RPMI 1640 culture medium, supplemented with 0.5% bovine serum albumin.

Application of Cyclic Mechanical Strain

Experiments testing the effects of mechanical strain were performed as previously described.[7] In brief, MCs were seeded into six-well, Flex I plates (5×10^4 cells/25-mm diameter well) with flexible collagen-coated bottoms from Flexcell International (McKeesport, PA) and cultured in a 1 ml/well of growth medium. After 24 hours, experimental cultures were subjected to cyclic stretching whereas the control cultures were maintained in a static environment under identical conditions. To mimic conditions of MC stretch during possible low-frequency oscillations in intraglomerular pressure, all experiments were performed using alternating cycles of 10 seconds of stretch and 10 seconds of relaxation (50 mHz).[28] Vacuum intensity was set to provide a 19% maximum elongation at the periphery of the well (~9% average elongation).

Adhesion Assay

MCs cultured on Flex I plates were washed after stretch or cytokine treatment and 8×10^5 neutrophils were added per well. After 1 hour at 37°C cultures were washed gently but extensively to remove the nonadherent cells. The remaining adherent cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, dried, then placed at −30°C until the time of staining. Cells were stained using Meyer's hematoxylin and the number of neutrophils per field was determined. At least nine fields per well were examined.

Analysis of ICAM-1 Expression

For analysis of mRNA, total RNA was isolated as previously described[29] and Northern blots hybridized and probed using ^{32}P-labeled cDNA of ICAM-1[30] or β-tubulin. Autoradiograms were digitized by scanning densitometry and quantified with image analysis.[7] The analysis of ICAM-1 protein expression was determined by immunostaining, using an alkaline-phosphatase avidin-biotin assay.[29] While attached to the supporting circular membrane, cells were fixed and pie-shaped wedges of this membrane were cut out. The primary antibody (1A29) used was ICAM-1-specific.[31] The slides were counterstained in Mayer's hematoxylin. Photographs were obtained at a ×100 magnification under identical illumination. To obtain the level of stain intensity, digitized 8-bit RGB color images were divided into quadrants, separately analyzed under identical density calibration, and the quantity of red color present determined using image analysis.

Statistical Analysis

Unless otherwise noted, differences between two groups were analyzed by the Student's paired *t*-test because of

the cloned nature of the MCs studied. In the case in which results were normalized to corresponding control values, the data were analyzed by a one-sample *t*-test with a hypothesized mean of 100% to compare the test group with the control. A two-sample, paired *t*-test was used to examine differences between multiple test groups. In both cases, a *post hoc* Holm's test was applied to adjust for multiple comparisons and the level of significance was set at 0.05.

Results

Effect of Stretch on ICAM-1 mRNA

To determine the level of constitutive MC ICAM-1 message and whether its expression is altered by cyclic strain, cells were exposed to various periods of stretching or allowed to remain under static conditions before harvesting cell layers for mRNA analysis. Control, unstretched MCs expressed detectable amounts of ICAM-1 message (Figure 1). Expression was markedly up-regulated with cyclic stretching. The induction was detected after 4 hours and progressively increased with longer periods of stretch, reaching 66 and 116% greater than the levels in static cultures at 8 and 12 hours, respectively (Figure 1).

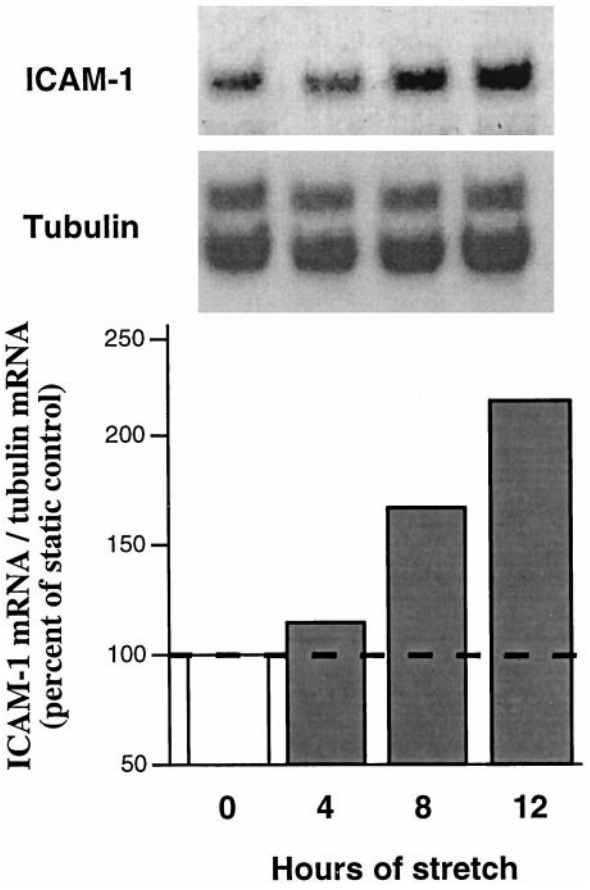

Figure 1. Time course for the effects of cyclic stretching on ICAM-1 transcript levels in MCs**. Top:** Northern analysis of mRNA for ICAM-1, or β-tubulin (from the same blot), after the indicated period of cyclic stretching. **Bottom:** Optical-scanning densitometry was used for the quantification of bands. Results shown were normalized to endogenous β-tubulin mRNA. Values are from a representative experiment. Four separate plates (24 wells) were pooled to make one sample.

Immunolocalization of ICAM-1 in MCs and the Effects of Mechanical Strain

Next determined was the level of ICAM-1 protein present in the MCs and whether the observed changes in ICAM-1 mRNA levels were associated with corresponding changes in protein expression. To accomplish this, MCs were fixed after cyclic strain or static incubation and the flexible substrates removed from the wells. Culture membranes were then immunostained for ICAM-1 protein. Because the force of elongation is not uniform across a stretched well, but increases from the center to the periphery, this attribute was used to determine the relationship between the amplitude of strain and the level of ICAM-1 present. Three assigned zones of the stretched cultures, experiencing 4%, 12%, and 18% elongation, respectively, were examined. Results were then compared to those from the outermost zone of static cultures. Static cultures showed weak staining in only a few MCs (Figure 2). In contrast, stretched MCs demonstrated localization of cell-associated ICAM-1 in amounts that were directly related to the increased elongation amplitude. To quantify this dose-related induction of ICAM-1 protein and to determine whether it was the result of a larger number of positive-staining cells, or a greater deposition of the molecule per cell, multiple fields were examined by image analysis. Figure 3A shows that although the number of positive-staining cells was near zero in static cultures, this number markedly increased in stretched cultures even in zones of minimal force. In areas of increasing amplitude of strain, there were corresponding increases in the number of positive-staining cells, reaching 90% in the zone of maximal stretch. Interestingly, the greatest change (1 to 50%) occurred in cells with lesser levels of strain (Figure 3A). When the intensity of staining per cell was analyzed, it increased significantly, even under minimal elongation and a dose response to increasing force also was evident (Figure 3B). However, in contrast to the proportion of positive staining cells, the cellular content of ICAM-1 demonstrated the greatest change at the higher levels of strain. Similar results were obtained when ICAM-1 expression was adjusted for the area of cytoplasm (Figure 3C).

Neutrophil Adherence in Response to Mechanical Strain

The observed up-regulation of both ICAM-1 mRNA and protein demonstrated a stretch-induced increase in production and cell-associated deposition. However, not determined was whether cyclic strain increased functional ICAM-1 on the cell surface, thereby increasing the interaction with leukocytes. To test this, we subjected MCs to either cyclic strain, or to TNF-α, a known stimulator of ICAM expression and ICAM-mediated leukocyte adherence. Freshly isolated human neutrophils were then

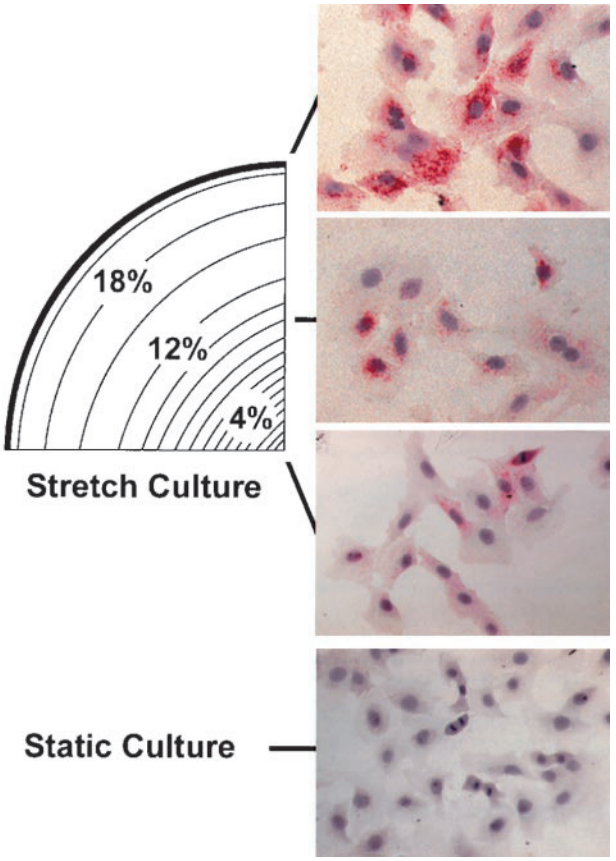

Figure 2. Immunostaining of MC-associated ICAM-1 under stretch or static conditions. Samples are from cultures of MCs either subjected to cyclic stretching for 8 hours or allowed to remain under static conditions. Red staining identifies reactivity of specific antibody with ICAM-1. Representative areas from outer, middle, and inner zones of a culture well membrane that are subject to decreasing amplitude of elongation are shown. Also shown is the outer zone of a static culture.

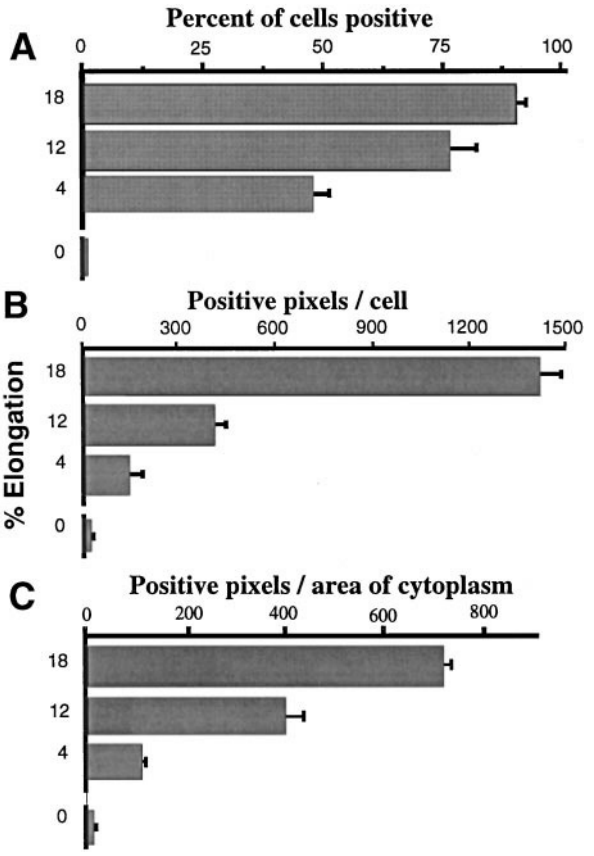

Figure 3. Effect of stretch amplitude on the quantity of cell-associated ICAM-1 protein. Quantitative image analysis measurements were obtained from the same experiments as depicted in Figure 2, in each of the indicated zones of elongation. Results are expressed as the percent of positive staining cells (20 or more red pixels per cell), the number of pixels per cell, or the number of pixels per area of cytoplasm. **Bars** indicate the mean of three separate examinations. Values at 0% elongation are from cells in the outermost zone of static cultures. **A:** Values from all stretched zones are significantly different from those in static culture, and the value from the outer most zone (18% elongation) is different from at that in the inner most zone (4% elongation) ($P < 0.05$). **B** and **C:** Values are all significantly different from each other ($P < 0.05$).

added and the number of attached cells was determined. As expected, treatment with TNF-α markedly stimulated neutrophil adherence (225% of control) (Figure 4). Cyclic stretch also potently induced leukocyte adherence, producing levels (282%) equal to or greater than that stimulated by TNF-α. MC cyclic stretch in the presence of TNF-α resulted in an additive effect on adherence (329%) that was significantly greater than that of TNF-α or stretch alone ($P < 0.05$).

Discussion

This study has shown for the first time that cyclic stretch up-regulates MC ICAM-1 transcript levels along with a rapid, concomitant increase in the corresponding protein. Further, the amount of ICAM-1 protein expressed was directly related to the amplitude of elongation. For example, whereas virtually no MCs stained for ICAM-1 in static cultures, ~50% were positive at a low 4% average elongation and increased to >90% positive at 18% elongation. Measurement of the amount of cell-associated ICAM-1 per cell showed that although relatively little force is required to induce the molecule, larger increases in expression require higher levels of elongation (18%). Importantly, our finding that the response was similar whether expressed per cell or per area of cytoplasm indicated that the observed effects were not the result of stretch-induced hypertrophy. The observed dose-response induction of ICAM-1 is similar to what we have previously reported for stretch-induced transforming growth factor-β, collagen I, fibronectin, and laminin,[7,29] whereas, the time course for induction of ICAM-1 mRNA and protein is similar to that reported by others in response to IFN-γ, TNF, and IL-1α.[23] The finding in nonstretched MCs of measurable constitutive ICAM-1 mRNA but little, or no, detectable ICAM-1 protein is not surprising. A strict correlation between the level of mRNA and the level of a corresponding protein, is not always the case.[32] Alternatively, this may simply reflect the differences in sensitivity of the two different assays.

The relevance of stretch-induced MC ICAM-1 expression to functional activity was demonstrated by a corresponding increase in ICAM-associated adherence. The same regimen of stretch used to induce expression of ICAM-1 produced a threefold increase in the adhesion of

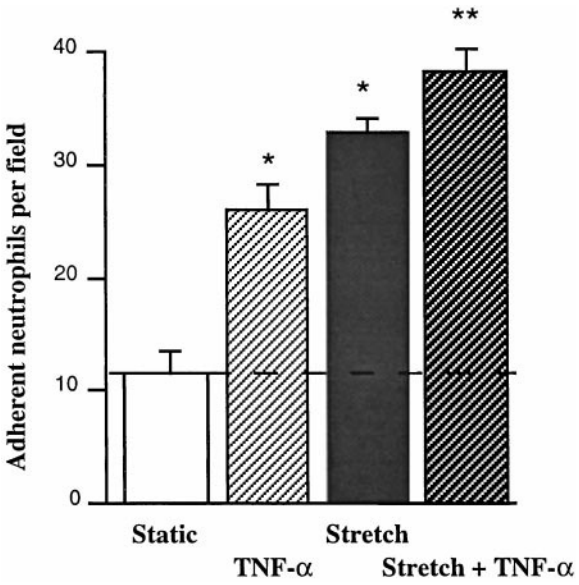

Figure 4. The effect of stretch and/or TNF on leukocyte adherence to MCs. Cultured MCs were exposed to the following conditions: control static, TNF-α (5 hours), cyclic strain (8 hours), or tumor necrosis factor-α (5 hours) in the presence of cyclic strain (8 hours). Human neutrophils were then added and after a 1-hour incubation period, the number of attached leukocytes was determined. The same areas were studied on each plate. The results are means from at least nine fields.

neutrophils. This stimulated adhesion was at least as great as that induced by exposure to TNF-α, a known inducer of MC ICAM.[23] It was interesting that an additive effect of stretch in combination with TNF-α was produced. This supports the idea that MC exposure to cytokines in combination with mechanical strain may form the basis for the accelerated development of glomerulosclerosis that occurs when intraglomerular hypertension and inflammation are simultaneously present. Although the leukocyte ligand for ICAM-1 was not determined in the present study, it has been previously shown that TNF- or IL-1-induced adhesion of MCs to phagocytes is abrogated by monoclonal antibodies directed against either MC ICAM-1, or leukocyte CD11/CD18.[14] In addition, we have previously reported that leukocyte-mediated, oxidant-induced injury of both human and rat (the same line used in the present study) MCs are also blocked by monoclonal antibodies to CD11/CD18.[16] Our findings in the present study with neutrophils are likely to be extended to monocyte/macrophage-MC interactions as well, because it has been previously shown that monocytes are also induced by TNF-α to up-regulate adherence to MCs via ICAM-1.[14]

The mechanism(s) for stretch-induced ICAM-1 expression is (are) unknown. However, in endothelial cells, the induction of ICAM-1 along with VCAM-1 and E-selectin, has been reported to be regulated at the level of gene transcription and to require binding of nuclear transcription factor κB to the respective promoters of these genes.[33] Further, TNF-α-induced ICAM-1 in mouse Sertoli cells seems to be mediated through the activity of a mitogen-activated protein kinase, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), but not p42/p44 or p38.[33] In MCs, the signaling pathways for mechanotransduction, including those leading to cytokine or ECM production, have not been clarified at this time. However, protein kinase C and protein tyrosine kinases are rapidly activated in MCs with stretch, followed by the induction of c-*fos* mRNA, and changes in this transcription factor have been linked to increased ECM production.[34,35] Mitogen-activated protein kinase is also activated by mechanical strain. One group has reported that both extracellular signal-related kinase and JNK were activated in a time- and intensity-dependent (10 to 30% elongation) manner.[36] However, another group reported that high levels of MC stretch (29%) activate p/44/42 and p38/HOG but not JNK, whereas the p38 activation is lost at reduced levels of stretch (20%).[37] Therefore, it remains to be determined whether the signaling pathways for stretch-induced ICAM-1 are like those for stretch-stimulated cytokines and/or ECM production, or for cytokine-induced ICAM-1.

Glomerular injury is a likely consequence of sustained up-regulation of ICAM-1. The possible mechanisms for ICAM-1-mediated damage are indicated by several recent studies. We have shown that phorbol myristate acetate-stimulated neutrophils adhere to cultured MCs through an ICAM-1 mediated interaction.[16] This stimulated adherence results in MC injury mediated by leukocyte oxidants, primarily H_2O_2. The requirement for physical contact was shown by the substantial blockade of stimulated cytolysis that occurred when adhesion was prevented by treatment with CD11/CD18 antibody.[16] In addition to these potentially lethal products, phagocytic leukocytes also produce inflammatory and prosclerotic cytokines including IL-1, IFN-γ, and transforming growth factor-β. These may act to stimulate MC growth, hypertrophy, and/or ECM metabolism subsequent to cell-cell interaction. Alternatively, monocytes are activated by soluble CD40 to induce MCs to produce high levels of MCP-1, IL-6, and to up-regulate ICAM-1.[38] The up-regulation of IL-6 and ICAM-1 required physical interaction of the two cell types. This indicates that an induction of ICAM-1 on MCs and their interaction with phagocytic leukocytes can lead to MC production of cytokines and chemokines capable of exacerbating glomerular inflammation. Nitric oxide also seems to be a product of phagocytic leukocyte/MC interaction, and may have both beneficial and damaging effects in the kidney. In large quantities it may have a direct toxic effect on MCs or act indirectly on the afferent arteriole and MCs to alter glomerular microcirculation.[39] Last, the up-regulation of ICAM-1 may directly alter MC-mediated effector function. MCs can function as antigen presenting cells for T-cell stimulation.[23] This requires stimulated expression of MHC class II molecules on the MCs as well as ICAM-1.[23] Holland and Owens[40] have recently shown that the cross-linking of ICAM-1 on a B lymphoma line induces a rapid increase in tyrosine phosphorylation of cellular proteins, the activation of the mitogen-activated protein kinase, and the up-regulation of class II major histocompatibility complex. This indicates that ICAM-1 functions not just as an adhesive molecule, but is also capable of transducing biochemical signals and suggests a mech-

anism for stretch-induced, ICAM-1-mediated antigen presentation by MCs. Our overall results *in vitro* are in agreement with the findings from recent studies of glomerular disease in both 5/6 nephrectomy[24] and in STZ-induced diabetic rat models.[21] They showed that the specific inhibition of ICAM-1 expression resulted in reduced leukocyte infiltration/proliferation and was associated with prevented deterioration of renal function.

In summary, these results demonstrate that cyclic stretch of cultured MCs produces a time- and dose-related up-regulation of ICAM-1 expression and a subsequent increase in neutrophil adherence. A sustained ICAM-1 up-regulation *in vivo* would therefore be expected, and explained, under conditions that produce increased glomerular size and/or pressure such as renal ablation, diabetes, or certain forms of glomerulonephritis. ICAM-1 could act downstream to mediate MC injury, enhance inflammation, and increase ECM accumulation. It may be that other cell types respond in a similar manner, thus facilitating their interaction as targets for leukocyte interaction. This could have important implications in many other diseases in which inflammation is the basis, including atherosclerosis.

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