Renal Proximal Tubular Epithelial Cell Transforming Growth Factor- β 1 Generation and Monocyte Binding

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With increasing awareness of the importance of renal cortical interstitial fibrosis, interest has focused on the mechanisms that stimulate generation of profibrotic factors including transforming growth factor (TGF)- β 1, by resident cells, such as proximal tubular epithelial cells (PTCs). Infiltration of monocytes, has been implicated in the pathogenesis of a wide variety of renal diseases, however, how interaction between monocytes and PTCs may affect the generation of TGF- β 1 by the resident cell is unknown. We demonstrate that monocytes stimulate TGF- β 1 transcription and protein synthesis by PTCs. This was dependent on direct cell contact and TGF- β 1 transcriptional activation that was dependent on ICAM-1 binding of unstimulated monocytes. This was mimicked by antibody cross-linking of PTC surface ICAM-1. We have previously identified hyaluronan (HA)-based structures on the surface of PTCs, both primary cultures and the HK-2 cell line. Removal of cell-surface HA increased ICAM-1-dependent monocyte binding and stimulation of TGF- β 1 synthesis. Furthermore, we demonstrate that binding of monocytes to HA-based structures on the cell surface of HK-2 cells interferes with this response. In summary, we have demonstrated that HA-based pericellular structures down-regulate proinflammatory and profibrotic responses by modulation of monocyte-driven ICAM-1-dependent cell activation and TGF- β 1 generation. (*Am J Pathol* 2004, 165:763–773)

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional cytokine that has been implicated in the pathogenesis of numerous fibrotic diseases. Its generation by proximal tubular epithelial cells (PTCs) suggests that this cell type may also influence pathological events in the renal interstitium. This is of particular significance because the rate of progression of renal disease is closely related to the degree of cortical interstitial fibrosis. Our interest has focused on the mechanisms that underlie the

generation of TGF- β 1 in the kidney and its role in progressive renal fibrosis. Inflammatory cell infiltration, and particularly monocyte/macrophage infiltration, has been implicated in the pathogenesis of a wide diversity of renal diseases.^{1–3} To date however, it is not known how monocyte-PTC interaction may regulate TGF- β 1 production or how this interaction may be regulated.

Recent studies *in vitro*, have shown that addition of exogenous hyaluronan (HA) oligosaccharides induces PTC expression of chemokines and leukocyte adhesion molecules.^{4,5} These findings suggest a role for HA in the pathogenesis of renal interstitial inflammation. HA is a ubiquitous connective tissue glycosaminoglycan, which *in vivo* is present as a high-molecular mass component of most extracellular matrices. Although HA is not a major constituent of the normal renal corticointerstitium,⁶ it is expressed around PTCs after renal injury caused by diverse diseases.^{7–10} Furthermore increased deposition of interstitial HA has been shown to correlate with both proteinuria and renal function in progressive renal disease.¹¹ The functional significance of these alterations in HA synthesis in the renal tubulointerstitium are, however, unclear.

We have previously examined the regulation of HA synthesis and also characterized the expression of the HA receptor CD44 in renal PTCs *in vitro*.^{12,13} More recently we have demonstrated that unstimulated PTCs form pericellular HA cable-like structures that bind mononuclear leukocytes via their cell-surface CD44 receptors.¹⁴ These observations are consistent with observations using colonic mucosal smooth muscle cells, which also demonstrated binding of nonactivated leukocytes to HA cable structures.¹⁵ Proximal tubular cells are known to express the adhesion molecules ICAM-1 and VCAM-1, and up-regulation of these adhesion molecules can predict outcome in inflammatory renal disease.¹⁶ In addition to demonstrating the formation of HA-based cables on unstimulated PTCs, we have shown increased monocyte binding after removal of HA cables using hyaluronidase, suggesting that HA cable-dependent monocyte binding may prevent their presentation to the cell-surface adhesion molecules.

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The aim of the study presented in this article was to examine the impact of monocyte-PTC interactions on the generation of TGF- β 1. The data demonstrate that binding of monocytes to PTCs ICAM-1 stimulates TGF- β 1 synthesis, and supports the hypothesis that HA cable-dependent monocyte binding blunts the profibrotic impact of the interaction of these two cell types.

Materials and Methods

Reagents and Antibodies

All tissue culture plastics including a cell-culture insert system (pore size, 1.0 μ m) were obtained from Becton Dickinson Ltd. (Oxford, UK). Reporter lysis buffer and Bright-Glo luciferase assay system were purchased from Promega (Madison, WI). FuGene6 transfection reagent was bought from Roche (Indianapolis, IN). ^{51} Cr as sodium chromate was obtained from Amersham Pharmacia, Biotech UK Ltd., (Buckinghamshire, UK). Ficoll-Paque Plus was from Amersham Biosciences (Bucks, UK). Other reagents and sources were as follows: testicular hyaluronidase (H3884) (Sigma, Poole, UK), recombinant human soluble ICAM-1 (R&D Systems Europe Ltd.), anti-human CD44 blocking monoclonal antibody BU75 (Ancell Corp., USA), anti-human CD18 blocking monoclonal antibody (Ancell Corp.), anti-human anti-CD54 (ICAM-1) cross-linking monoclonal antibody 6.5 (clone RR 6.5; a gift from Dr. R. Rothlein, Boehringer Ingelheim, Ridgefield, NJ), and anti-mouse IgG (whole molecule) (Sigma).

Cell Culture

All experiments were done using HK-2 cells (no. CRL-2190; American Type Culture Collection, Rockville, MD), which are human PTCs immortalized by transduction with human papilloma virus 16 E6/E7 genes.¹⁷ Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Biological Industries Ltd., Cumbernauld, UK), L-glutamine, insulin, transferrin, sodium selenite, hydrocortisone, and Hepes (Sigma-Aldrich, Poole, UK). Fresh growth medium was added to cells every 3 to 4 days until confluent. All experiments were done using cells at passage 30 or below, and cells were growth arrested in serum-free medium for 48 hours before use in experiments. All experiments were done in serum-free conditions.

U937 cells, originally derived from a human histiocytic lymphoma, were procured from the American Type Culture Collection. The cells were grown in suspension culture in RPMI medium supplemented with L-glutamine and penicillin/streptomycin, and containing 5% fetal calf serum. Cells were routinely subcultured at a 1:5 ratio three times per week. To ensure general applicability of the results obtained using the U937 cells, cell binding was also assessed using human mononuclear cells prepared by dextran sedimentation and Ficoll-Paque density separation as previously described.¹⁸

Analysis of TGF- β 1 Transcriptional Activity

The TGF- β 1 promoter-luciferase construct pGL3-TGF β 1 +11/-1362 was generated as previously described.¹⁹ The pSV- β -galactosidase control vector was purchased from Promega. For transfection of the reporter construct, 1.3×10^4 HK-2 cells were seeded per 24-well plate and incubated overnight. This density of cells produced a 70% confluent monolayer the following day. The cells were then transfected with 0.2 μ g of plasmid pGL3-TGF- β 1 +11/-1362, and 0.2 μ g of pSV- β -galactosidase plasmid (to act as an internal control for transfection efficiency), using the mixed lipofection reagent FuGene 6 (Roche) at a ratio of 3 μ l of FuGene to 1 μ g of DNA in serum-free and insulin-free medium. Twenty-four hours after transfection, unstimulated U937 cells were added to the monolayer of HK-2 cells for up to 24 hours. After lysis of the cells in reporter lysis buffer (Promega) β -galactosidase activity was determined by colorimetric assay (β -Galactosidase Enzyme Assay System, Promega), and luciferase content was quantified by glow-type luminance assay (Bright-Glo, Promega).

TGF- β 1 Protein Quantification

In all experiments total TGF- β 1 in the cell culture supernatants was measured by specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Active TGF- β 1 is measured directly and latent TGF- β 1 can be measured indirectly after acid activation of samples. This assay has <1% cross-reactivity for TGF- β 2 and TGF- β 3.

Assay for TGF- β 1 Activity

Activity of TGF- β 1 was examined by determining the luciferase activity of HK-2 cells transiently transfected with a Smad-responsive promoter construct as we have previously described.²⁰ The SMAD-responsive promoter (SBE)₄-Lux, was a gift from Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden).²¹

Conditioned medium was generated by addition of unstimulated U937 cells to HK-2 cells for 24 hours. Subsequently either untreated conditioned medium or conditioned medium subjected to 10 cycles of freeze thawing were added to cells transfected with the Smad reporter construct 12 hours before determining luciferase activity. Repeated cycles of freeze thawing of samples are well established *in vitro* mechanisms of activation of latent TGF- β 1.²²

Assay for Leukocyte Adhesion

U937 cell adhesion was measured as previously described.²³ Briefly HK-2 cells were grown on 24-well culture plates until confluent. On the day of assay, U937 cells (up to 70×10^6 cells/ml) were labeled for 90 minutes at 37°C with 100 μ Ci of ^{51} Cr as sodium chromate (Amersham BioSciences, Chalford St. Giles, UK). The labeled cells were washed three times with serum-free culture medium, counted on a hemacytometer, and resus-

pendent to 10^6 viable cells/ml (as determined by Trypan blue dye exclusion). Incubation medium was removed from HK-2 cultures, and 10^6 monocytes were added to each well. The binding phase of the assay was done at 4°C for 1 hour. All cultures were washed with cold medium before lysis by 1% Triton X-100. An aliquot was subsequently removed for quantitation of radiolabel. The number of the U937 cells bound per well was calculated from the initial specific activity (cpm/cell). Spontaneous release of chromium from U937 cells in control incubations without HK-2 cells was typically less than 10%.

Immunocytochemistry

Immunocytochemistry was done on cells grown in eight-well glass chamber slides (Nunc, Gibco/BRL Life Technologies Ltd., Paisley, UK). Cells were grown to confluence and incubated in serum-free medium alone for a further 24 hours. Culture medium was subsequently removed and the cell monolayer washed with sterile phosphate-buffered saline (PBS). Cells were fixed by addition of 100% ice-cold methanol for 15 minutes at -20°C. After fixation, cells were blocked with 50% fetal calf serum for 1 hour before a further washing step with PBS. For HA staining, a biotinylated HA-binding protein (b-HABP, 5 μ g/ml) was then added (Seikagaku Corp., Tokyo, Japan). Slides were incubated with b-HABP at 4°C overnight. The slides were washed with PBS before incubation with fluorescein avidin-D (20 μ g/ml) (Vector Laboratories, Burlingame, CA) used for visualization of b-HABP at room temperature for 1 hour. After a final washing step, specimens were affixed to the slides in Vectashield mounting medium (Vector Laboratories), and analyzed by confocal laser-scanning microscopy (TCS-40; Leica Microsystems, Cambridge, UK).

Statistical Analysis

Statistical analysis was performed using the unpaired Student's *t*-test, with a value of $P < 0.05$ considered to represent a significant difference. The data are presented as means $\pm$ SD of *n* experiments. For each individual experiment the mean of duplicate determinations was calculated.

Results

Addition of Monocytes Increased TGF- β 1 Synthesis

Transcriptional Activation of TGF- β 1 Promoter

The effect of co-culture of monocytes and PTCs on PTC TGF- β 1 gene transcription was assessed by addition of U937 cells to HK-2 cells transfected with a TGF- β 1 promoter-luciferase construct as described in Materials and Methods. Transfection reactions were performed in 24-well plates. In control cultures, serum-free medium alone was added to the transfected HK-2 cells. Initially TGF- β 1 promoter activity was assessed after addition of

Figure 1. U937 cell-mediated stimulation of TGF- β 1 promoter activity. Seventy percent confluent monolayers of HK-2 cells, seeded onto 24-well plates, were transfected with the TGF- β 1 promoter-luciferase construct. **A:** Twenty-four hours after transfection, 0 to 0.6×10^6 unstimulated U937 cells were added to each well in serum-free medium. After a further 24-hour incubation, luciferase activity was quantified and results expressed as the relative luciferase activity above that seen in transfected HK-2 cells to which no U937 cells were added. **B:** Time-dependent effect of U937 cells on TGF- β 1 promoter activity was assessed by addition of 0.3×10^6 cells to transfected HK-2 cells (solid bars). In control experiments serum-free medium only was added to the HK-2 cells (stippled bars). In addition, the effect of direct cell contact was examined by addition of U937 cell-conditioned medium (cross-hatched bars). TGF- β 1 promoter activity is expressed as the relative change in luciferase activity compared to the serum-free control at each time point. Results represent mean $\pm$ SD; $n = 6$; *, $P < 0.05$; **, $P < 0.005$ compared to control value at the same time point.

increasing numbers of U937 cells added to cultures of the transfected HK-2 cells, and incubated for 24 hours. The results of this experiment, shown in Figure 1A, demonstrated a dose-dependent increase in relative luciferase activity. Lack of toxicity to HK-2 cells was confirmed over the range of added monocyte number by quantitation of viable cells (data not shown). In subsequent experiments TGF- β 1 promoter activity was assessed after addition of 0.3×10^6 U937 cells, which gave the maximum response (Figure 1A). Using this standardized number of unstimulated U937 cells, a time-dependent increase in relative luciferase activity was observed, suggesting activation of TGF- β 1 gene transcription (Figure 1B). Twelve hours after the addition of monocytes, this represented a 1.5 ± 0.06 -fold increase greater than the control value (mean fold increase greater than control $\pm$ SD, $P = 0.0001$, $n = 6$).

Quantification of TGF- β 1 Protein

HK-2 cells were grown to confluence in 24-well plates. Subsequently 0.3×10^6 unstimulated U937 cells were added in the absence of serum. Cells were co-cultured for a further 72 hours, and the supernatant collected. Samples were centrifuged (4000 rpm for 4 minutes) to remove monocytes, and TGF- β 1 concentration in the supernatant was determined by ELISA (Figure 2). After addition of U937 cells, there was a time-dependent increase in TGF- β 1 protein, which was statistically significant at times longer than 24 hours of co-culture as compared to controls (control 121.6 ± 6.6 pg/ml versus U937 cells 150.0 ± 8.1 pg/ml, mean $\pm$ SD, $P = 0.0003$, $n = 5$). In control wells, confluent growth-arrested HK-2 monolayers were exposed to serum-free medium alone.

Differences in the quantity of TGF- β 1, as assessed by ELISA, were only detected after acidification of the samples, thus suggesting that TGF- β 1 was produced in its

Figure 2. U937 cell-mediated stimulation of latent TGF- β 1 protein synthesis. **A:** Confluent monolayers of HK-2 cells, grown on 24-well plates, were serum deprived before addition of 0.3×10^6 U937 cells per well, under serum-free conditions (solid bars). In addition serum-free medium alone (stippled bars) or U937 cell-conditioned medium (cross-hatched bars) were added to confluent monolayers of HK-2 cells. TGF- β 1 protein in the cell culture supernatant was quantified by ELISA at each of the time points indicated. Results represent TGF- β 1 concentration (pg/ml) $\pm$ SD; $n = 5$; $P < 0.005$ compared to control at each time point. **B:** Conditioned medium (CM) collected from HK-2 cells to which 0.3×10^6 U937 cells were added for 24 hours was added to HK-2 cells transfected with a Smad-responsive promoter-luciferase construct, (SBE) $_4$ -Lux. In addition, to activate latent TGF- β 1, conditioned medium was subjected to 10 cycles of freeze thawing (CM-FT) before addition to transfected cells. In control experiments, culture medium alone was added to the transfected cells. Luciferase activity was quantified 12 hours after the addition of conditioned medium. Results represent means $\pm$ SE of four individual experiments.

latent form. Further confirmation of the latency of TGF- β 1 was sought by addition of conditioned medium from HK-2 exposed to U937 cells, to HK-2 cells transiently transfected with the Smad-responsive promoter-luciferase construct. No difference in luciferase activity of the promoter construct was seen after addition of the conditioned medium (Figure 2B). In contrast, addition for conditioned medium that had been exposed to repeated cycles of freeze thawing, which leads to *in vitro* activation of latent TGF- β 1,²² led to a significant increase in Smad-responsive promoter activity.

TGF- β 1 produced by 0.3×10^6 unstimulated U937 cells in 24-well cultures in the absence of HK-2 cells was also measured. Monocytes were incubated in serum-free medium for 48 hours to mimic conditions of co-culture. Subsequently medium was collected and TGF- β 1 quantified. Under these conditions TGF- β 1 was undetectable by ELISA.

Increased TGF- β 1 Generation Required Direct Cell-Cell Contact

To determine whether the monocyte-dependent increase in TGF- β 1 expression was the result of the release of soluble factors from the monocytes or a result of interaction between the two cell-types, two different assays were used. The effect of U937 cell-conditioned medium on HK-2 cells was assessed by co-culture experiments in which the two cell types were separated by a permeable tissue culture insert (pore size, 1.0 μ m), and by medium transfer assays.

When HK-2 cells were grown to confluence on 24-well plates and U937 cells added to the apical aspect of a tissue culture insert, thus allowing co-culture in the absence of direct cell-cell contact, there was no significant increase in relative luciferase activity relative to the con-

Figure 3. Activation of the TGF- β 1 promoter requires cell-cell contact. **A:** HK-2 cells were transfected with the TGF- β 1 promoter-luciferase construct and 0.3×10^6 U937 cells were either added directly onto the HK-2 cell monolayer (contact, cross-hatched bars) or added onto a tissue culture insert (1.0 μ m pore size) to prevent direct cell-cell contact (insert, solid bars). In control experiments serum-free medium alone was added to the transfected HK-2 cell monolayer (control, stippled bars). After a further 24-hour incubation, luciferase activity was quantified and TGF- β 1 promoter activity was expressed as the relative change compared to the serum-free control. Results represent mean $\pm$ SD; $n = 6$; $P < 0.001$ compared to control. **B:** Conditioned medium (CM) collected from HK-2 cells to which U937 cells were added for 24 hours was added to HK-2 cells transfected with the TGF- β 1 promoter-luciferase construct-responsive promoter-luciferase construct. In addition, to activate latent TGF- β 1, conditioned medium was subjected to 10 cycles of freeze thawing before addition to transfected cells (CM-FT). In control experiments, culture medium alone was added to the transfected cells. Luciferase activity was quantified 12 hours after the addition of conditioned medium. Results represent means $\pm$ SE of four individual experiments.

rol value. In contrast when U937 cells were added to the basal compartment directly onto the HK-2 cell monolayer a significant increase in luciferase activity was seen (Figure 3A).

Similarly, addition of U937 cell-conditioned medium to HK-2 cells transfected with the TGF- β 1 promoter luciferase construct did not result in a significant increase in luciferase activity greater than the control value (Figures 2 and 3B). In contrast when the conditioned medium was subjected to repeated cycles of freeze thawing to activate TGF- β 1, a significant increase in TGF- β 1 promoter activity was demonstrated. These data confirm the need for direct cell-cell contact and demonstrate that no soluble factors are generated and released by either cell type on direct binding, which mediate transcriptional activation of TGF- β 1.

Removal of Cell-Surface HA Increases TGF- β 1 Expression in U937-HK-2 Co-Cultures

The relationship between monocyte-stimulated PTC TGF- β 1 synthesis and HA-dependent monocyte binding was examined by removal of HA from the surface of confluent monolayers of HK-2 cells with hyaluronidase before the addition of U937 cells. HK-2 cells were treated with bovine testicular hyaluronidase at a final concentration of 200 μ g/ml at 37°C for 10 minutes. Loss of HA cables in hyaluronidase-treated HK-2 cultures as compared to control cultures, was confirmed by confocal imaging (Figure 4A). HA was identified with a biotinylated preparation of the HA-binding protein and detected with fluorescent avidin-D (green).

Figure 4. Disruption of cell-surface HA increases TGF- β 1 promoter activity. **A:** Visualization of cell-surface HA. Confluent monolayers of HK-2 cells were serum deprived for 48 hours before fixation with methanol or treated with bovine testicular hyaluronidase (final concentration, 200 μ g/ml) at 37°C for 5 minutes before fixation and detection of HA by addition of biotinylated HA-binding protein. Confocal microscope images of the monolayers were collected ($\times 10$ objective). **B:** The influence of hyaluronidase on monocyte-dependent TGF- β 1 promoter activity. HK-2 cell monolayers were transfected with the TGF- β 1 promoter-luciferase reporter construct. Unstimulated U937 cells (0.3×10^6) were added to bovine testicular hyaluronidase-treated HK-2 monolayers (Hyal + U937) or to untreated monolayers (U937). In control cultures serum-free medium alone was added to either hyaluronidase-treated HK-2 monolayers (Hyal) or untreated monolayers (control). TGF- β 1 promoter activity was expressed as the relative change in luciferase activity compared to the serum-free nonhyaluronidase control. Results represent mean $\pm$ SD, $n = 9$.

The role of HA-dependent monocyte binding on TGF- β 1 expression was examined by addition of U937 cells to HK-2 cells transfected with the TGF- β 1 promoter-luciferase construct pretreated with hyaluronidase (Figure 4B). This resulted in a 1.89 ± 0.2 (mean $\pm$ SD, $n = 9$)-fold increase in relative luciferase activity greater than control value, compared to a 1.48 ± 0.139 (mean $\pm$ SD, $n = 9$)-fold increase when U937 cells were added to transfected HK-2 cells that had not been pretreated with hyaluronidase ($P = 0.0004$).

Interference of U937 Cell Adhesion to HA Increases TGF- β 1 Expression

Previously, we demonstrated that binding of monocytes to HA cables is CD44-dependent and could be blocked by addition of monocytes to HK-2 cells in the presence of a blocking antibody to CD44.¹⁴ To further examine the role of HA-dependent monocyte binding on TGF- β 1 expression, U937 cells were added to HK-2 cells trans-

Figure 5. Disruption of CD44-mediated U937 cell interaction with HA increases TGF- β 1 promoter activity. U937 cells (0.3×10^6) pretreated with increasing concentration of blocking antibody to CD44, at 37°C for 60 minutes, were added to TGF- β 1 promoter reporter construct-transfected HK-2 cells (α CD44) in the presence of the blocking antibody. In control experiments U937 cells (0.3×10^6) were added to transfected HK-2 cells in the absence of any blocking antibody (control) or in the presence of 5 μ g/ml of irrelevant IgG1 antibody control (IgG). In an additional experiment U937 cells were pretreated with 5 μ g/ml of anti-CD44 antibody and either soluble 400 ng/ml ICAM-1 (+ICAM) or 10 μ g/ml anti-CD18 antibody (+CD18) for 1 hour at 37°C, before their addition to transfected HK-2 cells pretreated with blocking antibody to CD44. During the co-culture period, cells were incubated with the CD44 antibody and also either the soluble ICAM or anti-CD18 antibody. TGF- β 1 promoter activity was expressed as the change in relative value comparing antibody-treated U937 cells to those not treated with antibody. Results represent mean $\pm$ SD; $n = 6$; *, $P < 0.0005$, compared to the serum-free control.

fected with the TGF- β 1 promoter reporter construct in the presence of blocking antibody to CD44 (Figure 5). Inhibition of HA-CD44 interaction by this method led to a dose-dependent increase in relative luciferase activity greater than the control values from cultures in which U937 cells were added in the absence of blocking antibody. Addition of U937 cells to transfected cells in the presence of an irrelevant antibody control (IgG1, 5 μ g/ml) did not influence TGF- β 1 transcription. Collectively these data suggest that in the presence of HA, fewer monocytes can access the HK-2 cell surface.

Soluble HA Reduces Monocyte Binding and Monocyte-Dependent TGF- β 1 Promoter Activity

The relationship between HA and epithelial cell-monocyte interaction was further examined by determining the effect of addition of exogenous HA on both monocyte binding and monocyte-dependent TGF- β 1 promoter activity. Monocyte binding was examined by addition of radiolabeled U937, pretreated with an increasing concentration of exogenous HA (0 to 50 μ g/ml), to HK-2 cells and bound radioactivity quantified. Under these conditions a significant reduction in monocyte binding was only seen at the highest dose of HA (Figure 6A). In parallel experiments unlabeled U937 cells were added to HK-2 cells transfected with the TGF- β 1 promoter reporter construct. In contrast addition of exogenous HA to U937 cells before their addition to transfected HK-2 cells did

Figure 6. Exogenous HA inhibits monocyte binding and TGF- β 1 promoter activity. **A:** Quantitation of monocyte binding: confluent monolayers of serum-deprived HK-2 cells were washed with PBS before addition of 1×10^6 ^{51}Cr chromium-labeled U937 cells under serum-free conditions for 1 hour at 37°C in the presence of an increasing concentration (0 to 50 $\mu\text{g}/\text{ml}$) of exogenous HA (MW, 2×10^6). Quantitation of bound radioactivity was performed as described in Materials and Methods and results represent mean $\pm$ SD of six individual experiments. **B:** In parallel experiments 0.3×10^6 U937 cells were added to TGF- β 1 promoter reporter construct-transfected HK-2 cells in the presence of an increasing concentration of HA. TGF- β 1 promoter activity was expressed as the relative change in luciferase activity compared to the control in which serum-free medium alone was added to the transfected cells. Results represent mean $\pm$ SD, $n = 6$.

not lead to a significant decrease in promoter activity and even at the highest concentration of HA, luciferase activity was significantly greater than the control value (Figure 6B). These data would suggest a dissociation between HA-dependent monocyte binding and monocyte-driven TGF- β 1 promoter activity.

Monocyte-Driven Stimulation of TGF- β 1 Is Dependent on ICAM-1-Mediated Cell-Cell Contact

Our previous observations suggest that in addition to HA cable-mediated binding, monocytes also bind to HK-2 cells via the adhesion molecule ICAM-1. The role of ICAM-1 was examined by addition of U937 cells to transfected HK-2 cells in the presence of a blocking antibody to the leukocyte integrin CD18 that is a ligand for ICAM-1 (Figure 7A). U937 cells were incubated with 10 $\mu\text{g}/\text{ml}$ of anti-CD18 antibody for 1 hour at 37°C before addition to transfected HK-2 cells also in the presence of the antibody. This resulted in a significant decrease in relative luciferase activity. Addition of an equivalent concentration of anti-CD18 antibody to transfected HK-2 cells in the absence of U937 cells did not affect TGF- β 1 promoter activity (data not shown). Furthermore addition of 10 $\mu\text{g}/\text{ml}$ of IgG did not influence U937 cells stimulated TGF- β 1 promoter activity. To confirm that blockade of ICAM1-CD18 interaction reduced monocyte binding, radiolabeled U937 cells were added to HK-2 cells in the presence of an increasing concentration of sICAM (0 to 800 ng/ml) and bound radioactivity quantified. These experiments demonstrated a dose-dependent decrease in monocyte binding that was significant at doses of sICAM of 400 ng/ml or above (Figure 7B).

Inhibition of ICAM-1-CD18 interaction also resulted in a significant inhibition of synthesis of TGF- β 1 protein as assessed by ELISA of the supernatant from parallel experiments (Figure 7C). Addition of U937 cells to confluent monolayers of HK-2 cells in the presence of either anti-

Figure 7. Inhibition of ICAM-1-CD18 interaction abrogates TGF- β 1 generation after addition of U937 cells. **A:** Inhibition of TGF- β 1 promoter activity by CD18-blocking antibody. U937 cells, either untreated (U937) or pretreated with 10 $\mu\text{g}/\text{ml}$ of anti-CD18 antibody (+CD18) or irrelevant IgG1 control (+IgG) for 1 hour at 37°C were added to HK-2 cells transfected with the TGF- β 1 promoter-luciferase construct. In the control experiment, serum-free medium alone was added to the transfected cells. TGF- β 1 promoter activity was expressed as the relative change in value compared to the serum-free control. Results represent mean $\pm$ SD, $n = 6$. **B:** Inhibition of monocyte binding by sICAM: confirmation that inhibition of ICAM1-CD18 interaction reduced monocyte binding was demonstrated by addition of 1×10^6 ^{51}Cr chromium-labeled U937 cells under serum-free conditions for 1 hour at 37°C to confluent monolayers of HK-2 cells in the presence of an increasing concentration (0 to 800 ng/ml) soluble ICAM. Quantitation of bound radioactivity was performed as described in Materials and Methods and results represent mean $\pm$ SD of six individual experiments. **C:** Inhibition of TGF- β 1 protein synthesis by blocking CD18. U937 cells, either untreated (stippled bars) or pretreated with 10 $\mu\text{g}/\text{ml}$ of anti-CD18 antibody (solid bars) or irrelevant IgG1 control (cross-hatched bars) for 1 hour at 37°C were added to confluent monolayers of HK-2 cells and TGF- β 1 quantified by ELISA at the indicated time points. Results represent mean $\pm$ SD, $n = 5$. **D:** Addition of U937 cells to the basolateral aspect of HK-2 cells increases TGF- β 1 transcription. HK-2 cells were subcultured and seeded onto the bottom side of polycarbonate tissue culture inserts (pore size, 3.0 μm). In the inverted position the cells were allowed to attach for 4 hours at 37°C, before inversion into six-well plates containing tissue culture medium supplemented with 10% fetal calf serum for a further 10 hours. Subsequently cells were transfected with the TGF- β 1 promoter-luciferase construct and 0.3×10^6 U937 cells were added directly into the insert thus accessing the basolateral aspect of the HK-2 cells in the absence (basal monos, solid bar) or presence of 10 $\mu\text{g}/\text{ml}$ of anti-CD18 antibody (basal monos + CD18, cross-hatched bar). In control experiments serum-free medium alone was added to the transfected HK-2 cell monolayer (control, stippled bars). After a further 24-hour incubation, luciferase activity was quantified and TGF- β 1 promoter activity was expressed as the relative change compared to the serum-free control. Results represent mean $\pm$ SD, $n = 6$.

CD18 antibody (10 $\mu\text{g}/\text{ml}$) or sICAM (400 ng/ml) led to a significant decrease in monocyte-dependent stimulation of TGF- β 1 protein concentration. Addition of IgG did not affect monocyte-dependent stimulation of TGF- β 1 generation.

Although ICAM-1 is predominantly expressed on the apical aspect of PTCs numerous studies have demonstrated expression of ICAM-1 on their basolateral aspect in renal disease.^{16,24–26} To demonstrate the relevance of our findings to the *in vivo* situations, HK-2 cells, transfected with the TGF- β 1 promoter-reporter construct were grown on the bottom side of permeable tissue culture insert (pore size, 3.0 μm), and monocytes added to the apical chamber of the insert to access the basolateral

Figure 8. Removal of cell-surface HA increases ICAM-1-dependent TGF- β 1 promoter activity. HK-2 cells transfected with the TGF- β 1 promoter-luciferase construct were treated with bovine testicular hyaluronidase (final concentration, 200 μ g/ml) at 37°C for 5 minutes before addition of U937 cells either alone (U937 + Hyal) or in the presence of either 400 ng/ml of soluble ICAM-1 (U937 + Hyal + sICAM-1), 5 μ g/ml of irrelevant IgG1 antibody (U937 + Hyal + IgG), or 10 μ g/ml blocking antibody to CD18 (U937 + Hyal + CD18Ab). In control experiments U937 cells were added to transfected HK-2 cells that had not received hyaluronidase treatment (control U937). TGF- β 1 promoter activity was expressed as the relative change compared to the value obtained when U937 cells were added to the untreated HK-2 cells. Results represent mean $\pm$ SD, $n = 6$.

aspect of the cells.²⁷ Using this method addition of U937 cells to the basolateral aspect of the HK-2 cells led to a significant increase in relative luciferase activity greater than the control value (Figure 7D). This increase was abrogated by incubation of the U937 cells on the basolateral aspect of the HK-2 cells in the presence of anti-CD18 antibody (10 μ g/ml).

The Relationship between HA and ICAM-1-Dependent Monocyte Binding and TGF- β 1 Synthesis

Increased TGF- β 1 generation after addition of U937 cells to HK-2 cells in which cell-surface HA was removed by hyaluronidase suggested that HA cable-dependent monocyte binding competed for and reduced ICAM-1-dependent cell activation. To explore this possibility, we examined the effect of inhibition of ICAM-1-CD18 interaction on the increase in monocyte-induced TGF- β 1 transcription in hyaluronidase-treated HK-2 cells. TGF- β 1 promoter-transfected HK-2 cells were treated with hyaluronidase. Subsequently U937 cells were added either in medium alone or in the presence of soluble ICAM-1 or blocking antibody to CD18. Inhibition of ICAM-1-CD18 interaction by either method prevented the increased relative luciferase activity seen after HA removal (Figure 8).

To relate alteration in TGF- β 1 promoter activity to changes in binding of monocytes, parallel experiments

Figure 9. Removal of cell-surface HA increases ICAM-1-dependent monocyte binding. Confluent monolayers of serum-deprived HK-2 cells were treated with bovine testicular hyaluronidase (final concentration, 200 μ g/ml) at 37°C for 10 minutes before addition of 1×10^6 ^{51} Cr chromium-labeled U937 cells. The role of CD18-ICAM-1 interactions in monocyte binding was determined by pretreatment of monocytes with 400 ng/ml of soluble ICAM-1 or 10 μ g/ml of blocking antibody to CD18 at 37°C for 1 hour before their addition to HK-2 cells in the presence of either blocking reagent. In control experiments U937 cells were added to untreated HK-2 cells. Quantitation of bound radioactivity was done as described in Materials and Methods. Data represent mean $\pm$ SD of six individual experiments.

were performed using radiolabeled monocytes to directly examine monocyte binding (Figure 9). Addition of either blocking antibody to CD18 or sICAM led to a significant reduction in bound radioactivity when radiolabeled U937 cells were added to HK-2 cells. Pretreatment of the HK-2 cell monolayer with testicular hyaluronidase (final concentration, 200 μ g/ml) at 37°C for 10 minutes before the addition of radiolabeled monocytes, led to a significant increase in bound radioactivity. This increase was abrogated by addition of the monocytes in the presence of either blocking antibody to CD18 or soluble ICAM-1, confirming that increased U937 cell binding was directly related to ICAM-1 adhesion. Furthermore, the increase in relative luciferase activity seen after disruption of HA-dependent monocyte adhesion to HK-2 cells transfected with the TGF- β 1 promoter luciferase construct, by addition of blocking antibody to CD44 antibody, was prevented by inhibiting ICAM-1-CD18 interaction with either soluble ICAM-1 or anti-CD18 antibody (Figure 5).

ICAM-1 Cross-Linking Increases TGF- β 1 in the Absence of Monocytes

To determine whether ICAM-1 ligation alone is sufficient for TGF- β 1 stimulation, ICAM-1 cross-linking experiments

Figure 10. ICAM-1 cross-linking increases TGF- β 1 promoter activity and protein synthesis. **A:** TGF- β 1 promoter activity after ICAM-1 cross-linking. **A:** HK-2 cells were transfected with the TGF- β 1 promoter-luciferase reporter construct, and subsequently cell-surface ICAM-1 was activated by tagging ICAM-1 on the cell surface with a specific monoclonal antibody (clone RR 6.5, 5 μ g/ml) and cross-linking by the addition of secondary anti-mouse antibody (5 μ g/ml). Subsequently, luciferase activity was quantified and TGF- β 1 promoter activity was expressed as the relative change compared to the value obtained without ICAM-1 cross-linking. Data represent mean $\pm$ SD; *, $P < 0.005$; $n = 6$. **B:** ICAM-1 cross-linking increases TGF- β 1 protein synthesis. ICAM-1 activation was done by tagging ICAM-1 on the cell surface with a specific monoclonal antibody and cross-linking by the addition of secondary anti-mouse antibody as described above in confluent monolayers of serum-deprived HK-2 cells (solid bars). Subsequently TGF- β 1 was quantified by ELISA at the time points indicated. In control experiments, either serum-free medium (stippled bars), or 5 μ g/ml of irrelevant IgG1 antibody (cross-hatched bar) were added to confluent HK-2 cell monolayer before quantification of TGF- β 1 by ELISA. Data represent mean $\pm$ SD, $n = 5$.

were done by a previously described method.¹⁸ ICAM-1 was tagged on the HK-2 cell surface with a specific monoclonal antibody and cross-linked with a secondary anti-mouse antibody. Cross-linking of ICAM-1 on TGF- β 1 promoter transfected HK-2 cells resulted in a time-dependent increase in relative luciferase activity similar to that seen after addition of U937 cells (Figure 10A). This became statistically significant after 6 hours of cross-linking. Adding irrelevant antibody followed by the secondary cross-linker did not influence TGF- β 1 promoter activity. This increase in promoter activity resulted in a significant increase in TGF- β 1 protein concentration in the supernatant fluid as assessed by ELISA, 48 hours after initiation of ICAM-1 cross-linking (Figure 10B).

Binding of Human Peripheral Blood Monocytes Increases HK-2 Cell TGF- β 1 Generation

Although U937 cells are a widely accepted model of monocytes, they were derived from a human histiocytic lymphoma. Therefore, to determine the scope and relevance of our observations, key experiments were performed with normal peripheral blood mononuclear leukocytes (PBMCs) in the place of U937 cells.

Addition of PBMCs resulted in significant stimulation of luciferase activity when added to HK-2 cells transfected with the TGF- β 1 reporter construct, similar to the results with the U937 cells (Figure 11A). This increase in TGF- β 1 promoter activity was significantly attenuated when the monocytes were co-cultured with transfected HK-2 cells but separated from them by a tissue culture insert. In addition increased promoter activity resultant from direct contact was inhibited by addition of the CD18 antibody confirming the involvement of the CD18-ICAM-1 interaction (Figure 11A). Furthermore inhibition of the interaction

Figure 11. Human peripheral monocytes behave in an identical manner to U937 cells, increasing TGF- β 1 promoter activity in an ICAM-1-dependent manner. **A:** Human peripheral monocyte increase in TGF- β 1 activity is dependent on cell-cell contact and CD18. HK-2 cells were transfected with the TGF- β 1 promoter-luciferase construct and 0.3×10^6 isolated human peripheral monocytes either added directly onto the HK-2 cell monolayer (contact, cross-hatched bars) or added onto a tissue culture insert (1.0 μ m pore size) to prevent direct cell-cell contact (insert, solid bars). In addition 0.3×10^6 human peripheral monocytes pretreated with 10 μ g/ml of anti-CD18 antibody (CD18) or irrelevant IgG1 control (IgG) for 1 hour at 37°C were added to transfected HK-2 cells. In control experiments serum-free medium alone was added to the transfected HK-2 cell monolayer (control, stippled bars). After a further 24-hour incubation, luciferase activity was quantified and TGF- β 1 promoter activity expressed as the relative change compared to the serum-free control. Data represent mean $\pm$ SD, $n = 6$. **B:** Removal of cell-surface HA increases human monocyte-dependent TGF- β 1 promoter activity. HK-2 cells transfected with the TGF- β 1 promoter-luciferase construct were treated with bovine testicular hyaluronidase (final concentration, 200 μ g/ml) at 37°C for 10 minutes before addition of human peripheral monocytes (0.3×10^6) either alone or in the presence of 400 ng/ml of soluble ICAM-1 or 10 μ g/ml of blocking antibody to CD18 (U939 + Hyal + CD18). In control experiments, U937 cells were added to transfected HK-2 cells that had not received hyaluronidase treatment. In parallel experiments, human peripheral monocytes were pretreated with 5 μ g/ml of anti-CD44 antibody alone or anti-CD44 antibody together with either soluble 400 ng/ml ICAM-1 or 10 μ g/ml anti-CD18 antibody for 1 hour at 37 °C, before their addition to transfected HK-2 cells (again in the presence of the relevant antibodies). After a further 24-hour incubation, luciferase activity was quantified and TGF- β 1 promoter activity expressed as the relative change compared to the value obtained when U937 cells were added to the untreated HK-2 cells. Data represent mean $\pm$ SD; $n = 6$; *, $P < 0.05$.

of PBMCs with HA, either by removal of cell-surface HA with testicular hyaluronidase, or by addition of monocytes in the presence of anti-CD44 antibody, increased TGF- β 1 promoter activity (Figure 11B). The increases in luciferase activity resultant from both hyaluronidase and CD44 antibody treatment were abrogated by inhibition of PBMC interaction with HK-2 cell-surface ICAM-1, which was achieved by use of either CD18-blocking antibody or soluble ICAM-1 (Figure 11B).

Discussion

TGF- β 1 is a key cytokine that has been implicated in the pathogenesis of progressive renal interstitial fibrosis. Much of our work to date has focused on the mechanism of its generation in the context of diabetic nephropathy. Elevated glucose concentration as seen in diabetes is known to stimulate TGF- β 1 synthesis within the glomerulus.²⁸ In contrast we have previously demonstrated that exposure of cultured human renal proximal tubular epithelial cells to elevated D-glucose concentrations increased the expression of a poorly translated TGF- β 1 transcript without any associated change in TGF- β 1 protein synthesis.²⁹ More recently we have demonstrated that pretreatment of PTCs with elevated glucose concen-

trations followed by stimulation with growth factors such as platelet-derived growth factor and interleukin-1 β had synergistic effects on TGF- β 1 synthesis.^{30,31} Although diabetes is primarily a metabolic condition, recent studies have also implicated macrophage infiltration in the pathogenesis of diabetic nephropathy. *In vivo* studies on streptozotocin-induced diabetic rats, have demonstrated prominent macrophage infiltration.^{1,3} In addition studies of renal biopsies taken from patients with type 2 diabetes have suggested that macrophages and their products are involved in the initiation of the pathological changes of human diabetic nephropathy.³² Macrophage influx has a broader application beyond the progressive cortical fibrosis of diabetic nephropathy in that macrophage influx is associated with progressive renal scarring in a wide range of disease states.^{33,34} These observations led us to examine the role of the interaction between monocytes and renal PTCs in the regulation of TGF- β 1 synthesis. The data demonstrate that such an interaction stimulated both transcription of the TGF- β 1 promoter, and also the synthesis of TGF- β 1 protein. Previously we have demonstrated that the TGF- β 1 transcript in PTCs is inherently poorly translated and that independent regulatory mechanisms exist for transcription and translation.^{20,31} These observations are therefore consistent with the relative delay between transcriptional activation and generation of TGF- β 1 protein in the current study. Although the majority of our observations were generated using U937 cells that were originally derived from a human histiocytic lymphoma, we have also demonstrated that the same phenomenon occurred after addition of human peripheral blood mononuclear leukocytes, thus demonstrating that this is not an effect confined to an abnormal, tumorigenic cell line.

An important observation from our data was the requirement for direct cell-cell contact to optimally stimulate TGF- β 1 synthesis. This was confirmed by the lack of effect of conditioned medium from either U937 cells or PBMCs on HK-2 cells. Furthermore, separation of the monocytes and epithelial cells with a permeable tissue culture insert also markedly attenuated this response. Interestingly however TGF- β 1 is produced predominantly in its latent form because it could only be detected by ELISA after acidification of the supernatant samples and similarly conditioned medium from glucose-stimulated cells was only able to stimulate a Smad-responsive promoter-luciferase construct after repeated cycles of freeze thawing. Both transient acidification and freeze thawing of samples are well-established *in vitro* mechanisms of activation of latent TGF- β 1.²² Drawing conclusions regarding the *in vivo* importance of this *in vitro* observation however is difficult because many mechanisms of *in vivo* activation related to proteolytic processing of the latent complex,^{35,36} and also conformation change of the latent complex, which may be mediated by integrin binding,³⁷ have been identified, and their regulation is beyond the scope of the data presented in this study.

Having demonstrated the importance of cell-cell contact in the regulation of TGF- β 1 synthesis, we subsequently identified ICAM-1-CD18 binding to be the critical determinant of this effect. This is consistent with numer-

ous studies that implicate ICAM-1 in the pathogenesis of progressive renal fibrosis. *De novo* tubular expression of ICAM-1 and increased expression by interstitial cells occurs in various human renal diseases, and in general, the intensity of staining correlates with disease activity.³⁴ Furthermore, administration of antibody to ICAM-1 has been proposed as a possible therapeutic option to preserve renal function at least in a model of ischemic renal injury.³⁸ Recent studies have highlighted ICAM-1-dependent binding of leukocytes to renal fibroblasts *in vitro*.³⁹ Furthermore direct contact of leukocytes with cell-surface fibroblast ICAM-1 led to cell activation and further up-regulation of ICAM-1 expression.¹⁸ These data suggested that renal cortical fibroblasts may be central to the control of renal interstitial inflammation, which is an important feature of progressive renal interstitial fibrosis regardless of its etiology. Although ICAM-1 is predominantly expressed on the apical aspect of PTCs numerous studies have demonstrated expression of ICAM-1 on their basolateral aspect in renal disease.^{16,24–26} Furthermore we have demonstrated that monocytes interacting with the basolateral aspect of HK-2 cells, stimulates TGF- β 1 promoter activity in an ICAM-1-dependent manner. This is therefore consistent with a potential *in vivo* role for polarized interactions between interstitial inflammatory cells and proximal tubular cells. Our data suggest that not only a fibroblast-leukocyte interaction, but also a PTC-leukocyte interaction, are important in regulating the response to infiltrating leukocytes.

In addition to characterizing the nature of the interaction between monocytes and PTCs that stimulated TGF- β 1 synthesis, the data presented also clearly demonstrate a role for HA in regulating this response. More specifically the data suggest that binding of monocytes to HA-based structures decreases monocyte activation of TGF- β 1 generation. This hypothesis is supported by the observation that disruption of CD44-HA interaction either by removal of cell-surface HA structures using hyaluronidase, or by addition of blocking antibody to CD44, increased monocyte binding and ICAM-1-dependent TGF- β 1 promoter activity and TGF- β 1 protein synthesis. We therefore suggest that HA cables keep monocytes away from ICAM-1 on cell surfaces, which would potentially prevent activation of both leukocyte⁴⁰ and the resident cell.¹⁸

CD44-HA interaction has been widely studied in the context of extravasation of leukocytes in vascular beds at the sites of inflammation. In contrast less is known regarding the role of HA-CD44 in the regulation of the interaction between leukocytes and resident extravascular cells such as PTCs. In the context of extravasation of leukocytes, many studies have demonstrated the need for activation of CD44 on the leukocytes before cell adhesion to blood vessel walls, which precedes leukocyte migration into the tissues.^{41–43} This is in contrast to our observation demonstrating binding of unstimulated monocytes via CD44 to renal epithelial cells. That unstimulated monocytes bind to HA-based structures further supports the hypothesis that this represents an anti-inflammatory response. This protective anti-inflammatory effect of HA is in marked contrast to the reported effect of

addition of exogenous HA, particularly addition of HA fragments of low molecular weight, which through their interaction with CD44 on PTCs participate in an amplification of recruitment and adhesion of leukocytes.^{4,5} We therefore postulate that soluble HA that interacts with CD44 on the epithelial cell, is functionally distinct from HA attached to the cell surface of the epithelial cell in cable-like configurations. Although the precise nature of the anchoring of the HA cables with the epithelial cell surface is not clear, it is not CD44-dependent. Studies in colonic smooth muscle cells and also our previous data demonstrate that assembly of HA-based cables is affected by the presence of blocking antibodies to CD44, although cell-surface HA patches are significantly diminished.¹⁵

In summary we have identified a novel mechanism regulating leukocyte-dependent proximal tubular cell activation. More specifically we have demonstrated that HA-based pericellular structures can bind leukocytes via a CD44-mediated interaction, and that this in turn blocks direct contact of the leukocyte with the surface of the epithelial cell preventing ICAM-1-CD18 interaction with consequent down-regulation of the proinflammatory and profibrotic response and subsequent TGF- β 1 generation.

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