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Invited Review

ICAM-1 interactions in the renal interstitium: A novel activator of fibroblasts during nephritis

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Summary. Chronic renal diseases often degenerate towards end-stage failure, requiring replacement renal therapy. The progressive decline of such diseases is a highly complex, multi-factorial process, which is poorly understood. Indeed, not all chronic conditions take on a progressive course, some may recover to regain normal function, while others may remain functionally impaired yet stable. The structural features of progressive decline, however, show common histological features, despite the diverse nature of the primary injury. These aberrant structural alterations are characterised essentially by a dramatic expansion of the tubulointerstitium, with accompanying tubular atrophy, resulting from interstitial fibrosis. These changes are thought to be a uniform response to prolonged inflammation which may originate in the glomerulus, the vasculature or the interstitial space (Strutz et al., 1995).

A histomorphometric analysis of renal diseases, initially performed by Risdon et al. (1968), and supported by Bohle et al. (1987) and others (Eknoyan et al., 1990), revealed that the severity of abnormal glomerular pathology did not always correlate directly with impaired renal function. The extent of interstitial inflammation and the degree of interstitial fibrosis, however, were both shown to be more accurate predictors of renal function (Bohle et al., 1992). Furthermore there was a high probability of irreversible functional decline, in the presence of interstitial fibrotic lesions and tubular atrophy. Interstitial fibrosis is therefore considered an important histological marker for end stage renal failure, and is believed to be functionally more significant than primary changes within the glomerulus.

In most tissues, resident fibroblasts are believed to be the cells principally responsible for the synthesis and breakdown of extracellular matrix (ECM) within connective tissues. Indeed in fibrotic diseases of lung and skin, the resident fibroblast has been identified as the most important cell responsible for the abnormal deposition of ECM components during the disease process (Phan et al., 1985). In the kidney, there are probably several sources of matrix components during fibrosis including tubular epithelial cells, inflammatory macrophages (Vaage and Linbland, 1990) as well as interstitial fibroblasts. Although the precise cellular source of the bulk of this matrix requires clarification, there is mounting evidence supporting a significant contribution from resident or infiltrating fibroblasts (Rodemann and Muller, 1990, 1991a,b; Strutz and Muller, 1995).

Key words: Renal fibrosis, Fibroblasts, ICAM-1, Cell adhesion

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Characterisation of renal cortical fibroblasts

Fibroblasts in a variety of fibrosed tissues, have been described as possessing some of the morphological and functional characteristics of smooth muscle cells. Initially described in granulation tissue of experimental skin wounds, these myofibroblastic cells, appeared transiently at the wound edge, and were characterised by the presence of α-smooth muscle actin (αSMA) positive cytoplasmic microfilaments (Darby et al., 1990). It was these myofibroblasts which were shown to provide the force for wound contraction (Skalli et al., 1989; Grinnell 1994). During the progression of pulmonary fibrosis, increasing numbers of myofibroblasts, at sites of fibrotic injury have been described (Khun and McDonald, 1991). Myofibroblastic cells have also been described in kidneys. During the progression of experimental nephritis (Zhang et al., 1995), there was an increasing myofibroblast population within the renal interstitium. Fibrotic progression, in this model, was associated with increased immunohistological staining for αSMA, both within the glomerulus and the interstitium, and these changes paralleled those of matrix deposition. Immunoelectron microscopy revealed a small population of fibroblastic interstitial cells which constitutively expressed αSMA in normal human kidneys (Alpers et

al., 1994). In 128 human renal biopsies, large accumulations of these myofibroblasts were seen at sites of tubulointerstitial injury, and these may thus represent the principal cells contributing to fibrotic disease.

No precise role for the resident population of cortical interstitial myofibroblasts has yet been described. The expression of αSMA however has recently been associated with the retardation of motility in fibroblasts (Ronnov-Jessen and Petersen, 1996). This protein may therefore mark the end of the migration of infiltrating fibroblasts. Although the presence of myofibroblasts suggests a possible contractile function *in vivo*, the evidence to support such a role is weak, and a function for such contraction in renal physiology remains to be identified. A contractile function for renal fibroblasts has recently been described, however, for fibroblasts isolated from the medullary interstitium. In response to vasoconstricting agents such as endothelin-1 and vasopressin, medullary interstitial fibroblasts from rats were shown to contract. The authors speculated that in the rat kidney the contraction of medullary fibroblasts may aid the concentration of urine, or be involved in tubular haemodynamics (Hughes et al., 1995). Regardless of a speculative contractile role, the increased cortical myofibroblastic population observed in fibrosed kidneys highlights possible parallels between wound healing and fibrotic diseases, and suggests that fibrosis itself is an attempt at healing, which has gone away.

The cellular origin of the increased myofibroblastic population in the diseased interstitium is not yet known. It is possible that during the progression of disease, various factors in the disease environment act to promote the proliferation of resident myofibroblasts, resulting in a local expansion of this population at sites of tubulointerstitial injury. Alternatively these factors may promote the migration of myofibroblasts, from the vasculature, for example, to sites of injury. Recently however, it has been suggested that cellular differentiation may contribute to this increased population. Transdifferentiation, where a cell looses the characteristics of one phenotype, while acquiring those of another can occur in a variety of cell types. For example, there is evidence to indicate that tubular epithelial cells can acquire fibroblastic characteristics (Hay, 1993). When grown in three dimensional culture, they lose epithelial markers, like cytokeratin, and gain fibroblast characteristics such as the expression of the intermediate filament vimentin. Furthermore, the expression by rodent tubular epithelial cells of a fibroblast specific protein (FSP-1) has been described *in vivo*, during late stage fibrosis, indicating that the transdifferentiation of epithelial cells into fibroblastic cells may indeed be possible (Strutz, 1995). Studies of hepatic fibrosis have shown that the perisinusoidal Ito cells (otherwise known as pericytes or lipocytes) lose their physiological vitamin A storing capacity, and assume myofibroblastic characteristics during progression. Such transdifferentiated lipocytes were shown to be the primary source of matrix production in liver fibrosis, with resident fibroblasts making little if any contribution (Friedman, 1993). Other cells such as vascular smooth muscle cells (Chamely-Campbell et al., 1979), and macrophages (Bhawan and Majo, 1989) have also demonstrated the ability to become fibroblastic. The study of such phenotypic plasticity remains poorly understood however, and the relative contributions made by such transdifferentiated cells to the overall fibrotic condition has not been addressed.

The understanding of the nature and role of the renal cortical fibroblast has been significantly hampered by difficulties in their isolation *in vitro*. The cortical interstitium comprises only a small fraction of the total cortical area, therefore, relative to other cell types, nephrectomy specimens yield few fibroblasts. Furthermore, the lack of fibroblast specific markers has made it difficult to ensure reliable characterisation. One approach taken to overcome problems of routine primary cell isolation was the viral transformation of isolated cell lines. This approach has allowed comparisons to be made with fibroblasts isolated from other tissues, such as skin (Lonnemann et al., 1994a,b). These studies have begun the preliminary analysis of renal fibroblast functions, and have demonstrated that the transformed cells synthesise and respond to cytokines that initiate a large number of morphological and functional changes. Many of the responses of the cells, however, are likely to be affected by the process of transformation. For example, transformed medullary fibroblasts from both normal and fibrotic kidneys lost their sensitivity to the proliferative effects of fetal calf serum and continued to proliferate in serum free conditions. In contrast, normal (non-transformed) skin fibroblast growth was absolutely dependent on serum in the culture medium. Thus transformed cell lines may be of limited use in the analysis of fibroblast responses, as they are innherantly abnormal, particularly with respect to factors involved in cellular activation and in cell proliferation (Wang et al., 1996).

Initial attempts, by our group, at isolating cortical fibroblasts in culture involved disrupting the renal cortex, through mechanical sieving and through enzymatic digestion of matrix components, as well as by modifying culture conditions such as serum concentration, and medium composition and supplements. These methods, however, served only to promote the outgrowths of epithelial cells. Occasionally, primary cell outgrowths were composed of several cell types, including epithelial, mesangial and fibroblastic cells. The epithelial cells usually predominated, and would invariably overgrow other cells present. The lack of a fibroblast specific marker made it impossible to identify or isolate fibroblastic cells present in such mixed cultures. The greatest success was achieved by cutting cortical tissue into small pieces followed by regular changes of culture medium. This approach yielded homogeneous primary fibroblastic cells in about 1 in 10 attempts. These cells, although morphologically similar, could be distinguished from mesangial cells, by their

inability to form the "hills and valleys" clusters typical of confluent glomerular mesangial cell cultures. The low success rate of this method is comparable to that reported previously for medullary fibroblasts (Rodemann and Muller, 1990, 1991a,b).

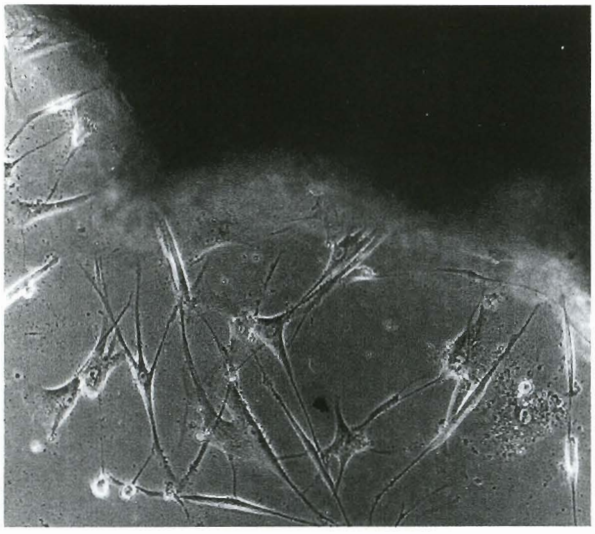
In culture, renal cortical fibroblasts exhibited a spindle shaped morphology similar to that of lung fibroblasts of comparable passage number (Fig. 1). In contrast to transformed cells (Lonnemann et al., 1994a,b) their proliferation required serum concentrations of >1%, and they typically underwent around 7 to 9 passages before becoming quiescent. At high passage numbers, morphological changes occurred which were associated with decreased proliferation and which may represent the mitotic to post mitotic phenotype change described for medullary fibroblasts by Muller and Strutz (1995).

Successful fibroblast cultures were examined by immuno-histology, for a broad range of intracellular and cell surface proteins. Kidney sections were stained in parallel with the same antibodies, which allowed direct comparisons to be made between fibroblasts *in vitro* and fibroblasts within the interstitium (Clayton et al., 1997).

The expression of CD90 (Thy-1) by fibroblasts in culture has been useful in distinguishing fibroblasts from other cell types which may be morphologically similar. CD90 was identified in sections of normal kidney on interstitial cells, proximal tubular epithelium and on the uroepithelial cells of Bowmans capsule (Fig. 2). In agreement with previous studies (Dalchau et al., 1989; Miyata et al., 1990; Hazen-Martin et al., 1993) no intraglomerular staining was observed. CD90 is an antigen that is present on a variety of tissues and shows considerable variation in distribution between species. For example, in contrast to human renal tissue it has been described principally as a marker of mesangial cells in rodents. It is a GPI-anchored protein of around 21 kD that is present on the cell membranes of brain cells (McKenzie and Fabre, 1981; Kemshead et al., 1982), and is thought to play a role in the transduction of proliferation signals (Gunter et al., 1987; Mayani and Landsdorp, 1994) and as a form of adhesion molecule (Morris and Ritter, 1980; Ritter and Morris, 1980; He et al., 1991; Hueber et al., 1992). To date, however, a definite function for the molecule in fibroblasts from the kidney has not been described.

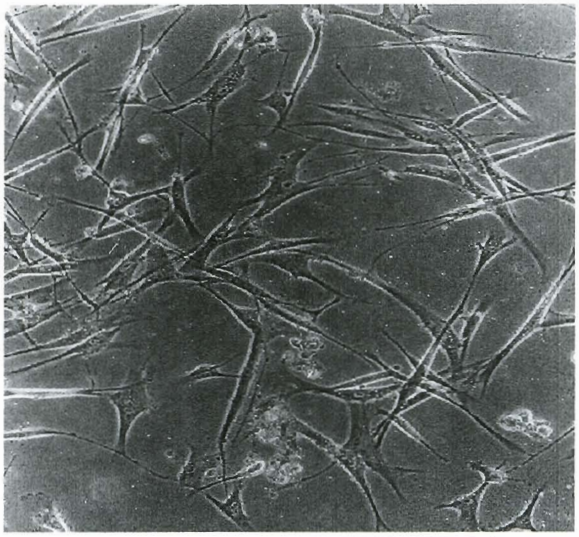
Cells isolated from the glomerulus and placed in culture did not express the CD90 antigen, and proximal tubular cells in primary culture lost their positive

A).



after 2 weeks in culture.

B).



C).

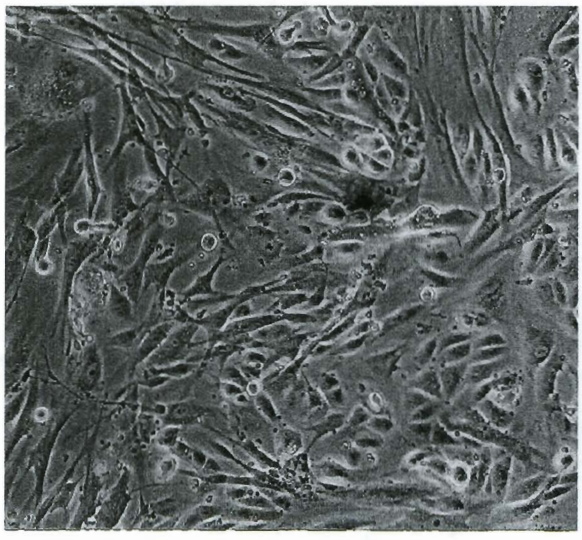


Fig. 1. Primary cultures of renal cortical fibroblasts. Cellular outgrowths from explanted tissue arise within 1-2 weeks **(A)**. In approximately 10% of attempts, homogeneous fibroblastic cultures were obtained **(B)**. In most cases however (70%), heterogeneous cultures were obtained, composed of fibroblasts and mesangial cells, but predominantly epithelial cells **(C)**. x 200

Table 1. Immunophenotyping of interstitial fibroblasts in culture.

	LUNG FIBROBLASTS	NORMAL RCF	FIBROTIC RCF
Vimentin	++++	++++	++++
α smooth muscle actin	-	++	+++
Desmin	-	-	-
Cytokeratin	-	-	-
Myosin	+++	+++	+++
Factor VIII	-	-	-
PDGF α	++	++	++
PDGF ß	++	++	++
ß1 integrin	+	+	+
ß3 integrin	+	+	+
α2 integrin	+	+	+
CD44	+++	+++	+++
CD45	-	-	-
CD90	+++	+++	+++

-: none; ±very weak; +: weakly positive; ++: positive; +++: strongly positive; ++++: very strong.

Table 2. Immunophenotyping of interstitial fibroblasts in tissue sections.

	NORMAL INTERSTITIAL CELLS	FIBROTIC INTERSTITIAL CELLS
Vimentin	+++	++++
α smooth muscle actin	+++	++++
Desmin	-	-
Cytokeratin	-	-
Factor VIII	-	-
ß1 integrin	+	+
ß3 integrin	+	+
α2 integrin	+	+
PDGFα receptor	++	++
PDGFß receptor	++	+++
CD44	+++	+++
CD45	-	-
CD90	+++	++++

-: none; ±: very weak; +: weakly positive; ++: positive; +++: strongly positive; ++++: very strong.

staining. Only fibroblastic cells retained the expression of CD90 in culture. Immuno-magnetic sorting based on CD90 expression thus proved a successful method for isolating fibroblasts from mixed cell populations. The CD90 positive fibroblasts isolated using this marker exhibited an identical profile of cell surface and cytoskeletal markers which we described for fibroblasts obtained without magnetic sorting. Although we saw homogeneous immuno-fluorescent staining for CD90, the possibility that a CD90 negative population exists in vivo can not be ruled out. Therefore fibroblasts isolated from mixed cultures by immuno-magnetic sorting may represent a subpopulation of $CD90^+$ cells.

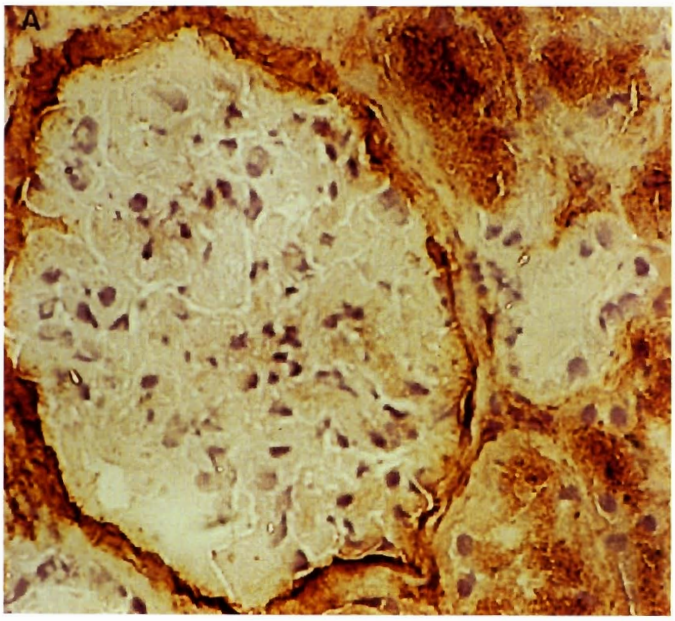


Fig. 2. Anti-CD90 staining of human renal cortex. Interstitial cells, tubular epithelial cells, and the uroepithelial cells of the bowmans capsule stain positively. Cells within the glomerulus are CD90 negative. x 310

Cultured renal cortical fibroblasts possessed a cytoskeletal protein profile typical of myofibroblasts. The lack of desmin expression indicated that their origin was likely not from smooth muscle cells (Sapino et al., 1990). Not all cells in a given population, however, exhibited myofibroblastic characteristics. Alpha-smooth actin expression for example was variable in culture. Such heterogeneity is not unexpected given the reported phenotypic complexity of fibroblast populations from other tissues (Desmouliere et al., 1992; Phipps, 1992). When derived from fibrosed renal tissue, however, the proportion of myofibroblastic cells in the population was dramatically increased (Tables 1, 2).

As well as intracellular proteins, we have demonstrated the expression of several cell surface receptors. Renal cortical fibroblasts express $ß_1$, $ß_3$ α_2 and α_4 integrin chains. The integrin ß chains associate with a variety of a chains, forming heterodimeric receptors capable of interacting with components of the extracellular matrix. For example the $\alpha_2ß_1$ dimer is a major receptor for binding collagen and laminin (Kupiec-Weglinski et al., 1993). Furthermore the cell surface hyaluronic acid receptor CD44 was also expressed. As well as a role in promoting cell adhesion to surrounding matrix, these receptors also play an important role in signalling, and in the regulation of matrix homeostasis or remodelling (Juliano and Haskill, 1993).

The expression of receptors for PDGF by interstitial fibroblasts has been demonstrated previously on kidney sections (Alpers et al., 1993, 1994). We also demonstrated that both PDGFα and PDGFß receptors are expressed by interstitial fibroblasts in culture. During inflammation, increased PDGF release, for example from platelets or by macrophages, may directly modulate fibroblast behaviour (Kovacs, 1991). PDGF is a potent

chemotactic factor for fibroblasts, promoting their migration and accumulation at sites of tissue injury (Knecht et al., 1991), and thus its actions may contribute directly to the progression of fibrosis.

The role of the renal cortical fibroblast in inflammation

Although there is evidence to indicate that inflammatory cells may contribute directly to matrix deposition (Vaage and Lindbland, 1990), the fibroblast is considered to be the principal source of the expanded extracellular matrix in fibrosis. The activation of fibroblasts into a vigorous fibrotic phenotype, however, is a poorly understood phenomenon. Several highly complex mechanisms are likely to contribute to the acquisition of disease characteristics. In the normal interstitium, fibroblast behaviour is regulated by many signals, originating from other resident renal cells, such as tubular cells, glomerular cells, vascular endothelial cells and tissue macrophages. In addition, fibroblast behaviour may be modulated through interactions with the surrounding extracellular matrix. These signals act in concert, to ensure appropriate matrix turnover and proliferation and therefore ensure homeostasis is maintained. In the inflamed interstitium, however, the nature of these signals may change. The activation of resident cells together with the infiltration of leukocytes into the interstitium, results in a markedly increased pool of potent cytokines, and growth factors, capable of directly modulating fibroblast behaviour. The breakdown products of ECM components, released as leukocytes infiltrate the tissues, may also provide a stimulus for fibroblast activation, possibly promoting subsequent synthesis of fibroblast matrix. In addition, other potential stimuli in the inflamed environment, may occur through direct cell-cell contact between resident fibroblasts and infiltrating inflammatory cells. The analysis of such potential interactions would be a novel approach to the study of renal fibrogenesis.

The role of intercellular adhesion

The expression of inflammatory cell adhesion molecules by cells other than endothelial cells has received increasing attention recently. During the inflammatory infiltration of tissues there are opportunities for direct interactions between resident tissue cells and leukocytes, mediated by specific adhesion molecules. These interactions may be involved in the regulation of inflammatory responses, with leukocytes being recruited, targeted and retained at appropriate sites.

Adhesion molecule expression during disease is upregulated on glomerular cells, tubular epithelial cells and interstitial cells (Muller et al., 1991; Bruijin and Daklo, 1993; Hill et al., 1994a) and the increase in ICAM-1 expression correlates particularly well with leukocyte infiltration, accumulation and immune mediated injury (Hill et al., 1994a). Two major members of the IgG superfamily of adhesion molecules (ICAM-1 and VCAM-1) were detected on cultured cortical fibroblasts, while the selectin-ELAM-1 was not expressed. Both ICAM-1 and VCAM-1 expression could be increased by exposure to proinflammatory cytokines such as TNFα. While the increased expression of these

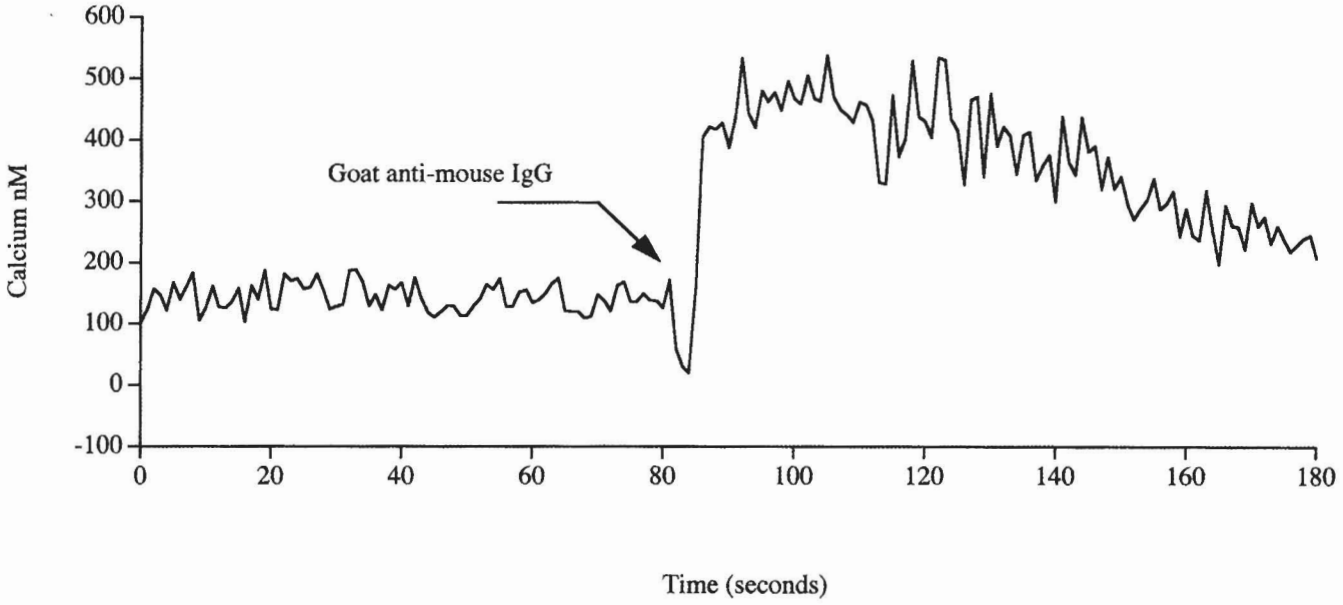
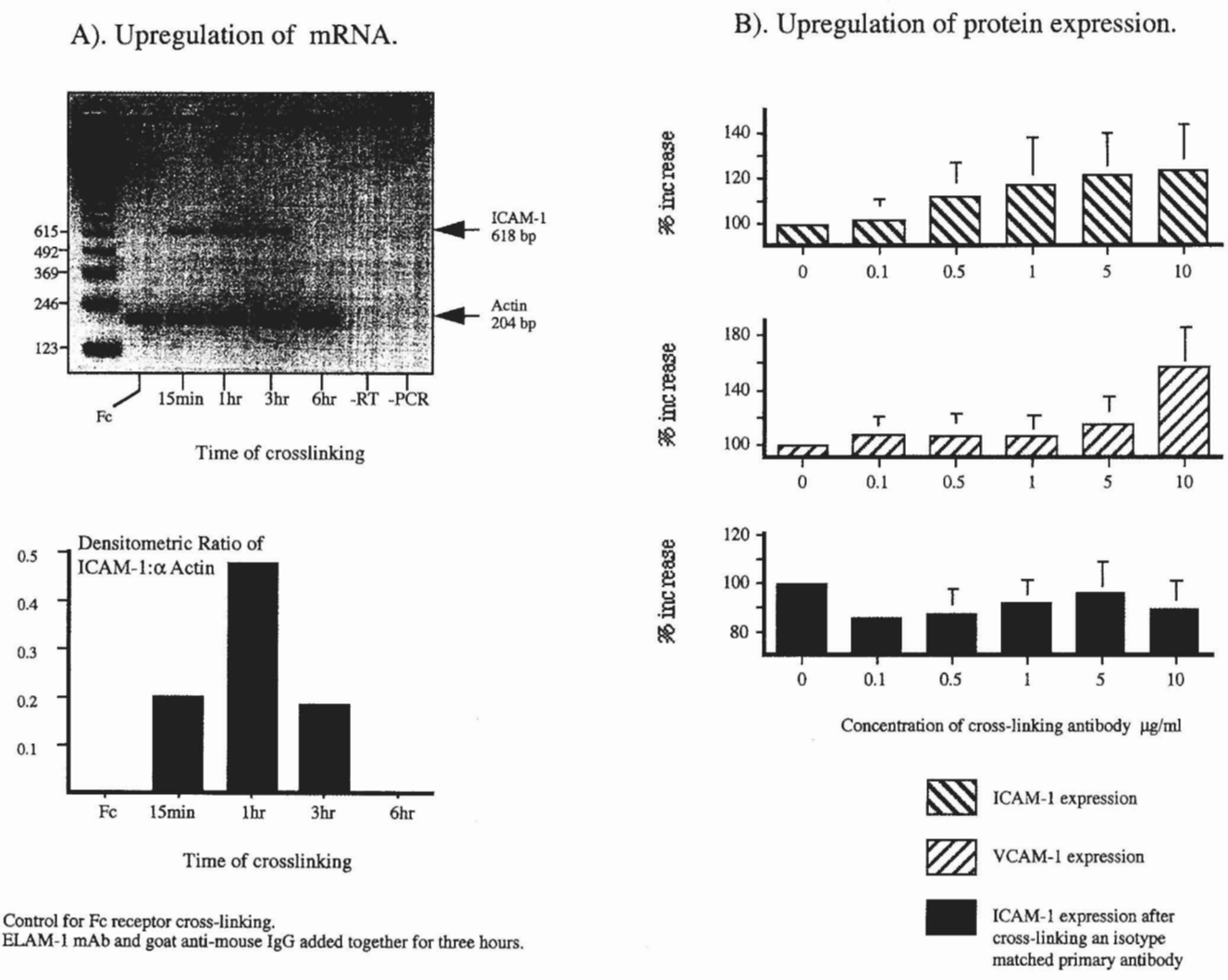


Fig. 3. Dualwavelength fluorometry with Fura-2-AM loaded fibroblasts. Cytokine treated fibroblasts (TNFα at 10^{-10}M for 24 hours) were incubated with anti-ICAM-1 antibody. Cross-linking antibody was then added (arrow), and the subsequent changes in intracellular free calcium were calculated from the 340/380 emission wavelength ratio.

molecules does not necessarily correlate with increased leukocyte binding (Piela and Korn, 1990), we demonstrated through leukocyte binding studies, that these molecules on RCF were functional (Clayton et al., 1997). With respect to the binding of mononuclear cells to fibroblast monolayers, we showed that the interaction of ICAM-1 with LFA-1 was more significant than the contribution of VCAM-1/VLA-4. This finding supports studies addressing the relative importance of ICAM-1 interactions *in vivo* (Bradey 1994; Hill et al., 1994a,b).

As a direct consequence of adhesion molecule ligation, changes in intracellular free calcium have been recorded in neutrophils (Jaconi et al., 1991; Peterson et al., 1993), endothelial cells (Huang et al., 1993; Pfau et al., 1995), and lymphocytes (Pardi et al., 1989). These calcium changes therefore represent early signalling events resulting from cell-cell contact. Through confocal imaging, we demonstrated that the adherence of neutrophils to fibroblasts elicited an increase in intracellular calcium in the fibroblasts (Clayton et al., 1998). The addition of cytokine-activated neutrophils to cytokine-activated fibroblast monolayers dramatically increased fibroblast cytosolic free calcium. Cytokine activation of both cell types was a prerequisite for intracellular calcium elevations. The treatment of endothelial cells or fibroblasts in other systems with proinflammatory cytokines increases the expression of adhesion molecules such as ICAM-1 and VCAM-1 (Krzeiscki et al., 1991; McEver, 1992; Bevilacqua, 1993; Tessier et al., 1993; Albelda et al., 1994; Cho et al.,



Fc = Control for Fc receptor cross-linking.
Anti ELAM-1 mAb and goat anti-mouse IgG added together for three hours.

Fig. 4. Effect of cross-linking ICAM-1 on the fibroblast surface. Cytokine treated fibroblasts were incubated with anti-ICAM-1 antibodies for 1 hour, followed by the addition of secondary cross-linking antibody (Goat anti-mouse IgG). Total RNA was isolated at times up to 6 hours. Reverse Transcription-PCR demonstrated an induction of ICAM-1 mRNA, reaching a maximum by 1 hour **(A)**. The ligation of ICAM-1 also resulted in the increased expression of cell surface adhesion molecules (ICAM-1 and VCAM-1), which was dose dependant on the concentration of cross-linking antibody **(B)**. Cross-linking an isotype matched antibody did not induce adhesion molecules.

1994). Similarly, treatment of neutrophils with TNF promotes their functional responses, and particularly leads to upregulation of integrin receptors such as LFA-1 and Mac-1 (Borregaard et al., 1994). Thus elevations in cytosolic calcium require activated levels of adhesion molecule expression by both leukocytes and their target cells. Such a mechanism may ensure that contact between cells which are not in an activated state does not result in comprehensive calcium-driven cell activation *in vivo*.

By mimicking leukocyte adhesion through cross-linking ICAM-1 using monoclonal antibodies, we demonstrated that the ligation of fibroblast ICAM-1 was one mechanism that would trigger elevations in intracellular calcium (Fig. 3). Furthermore, we demonstrated that most of this increase resulted from an influx of extracellular calcium. Thus the expression of ICAM-1 by fibroblasts promoted not only leukocyte adhesion, but also activated the cell through increasing the levels of intracellular free calcium.

Clearly ICAM-1 has the ability to transduce signals in fibroblasts upon leukocyte adhesion. The subsequent effect of this interaction on fibroblast behaviour is not a well investigated phenomenon. Bombara et al. demonstrated that the adhesion of lymphocytes to synovial fibroblasts induced cytokine and adhesion

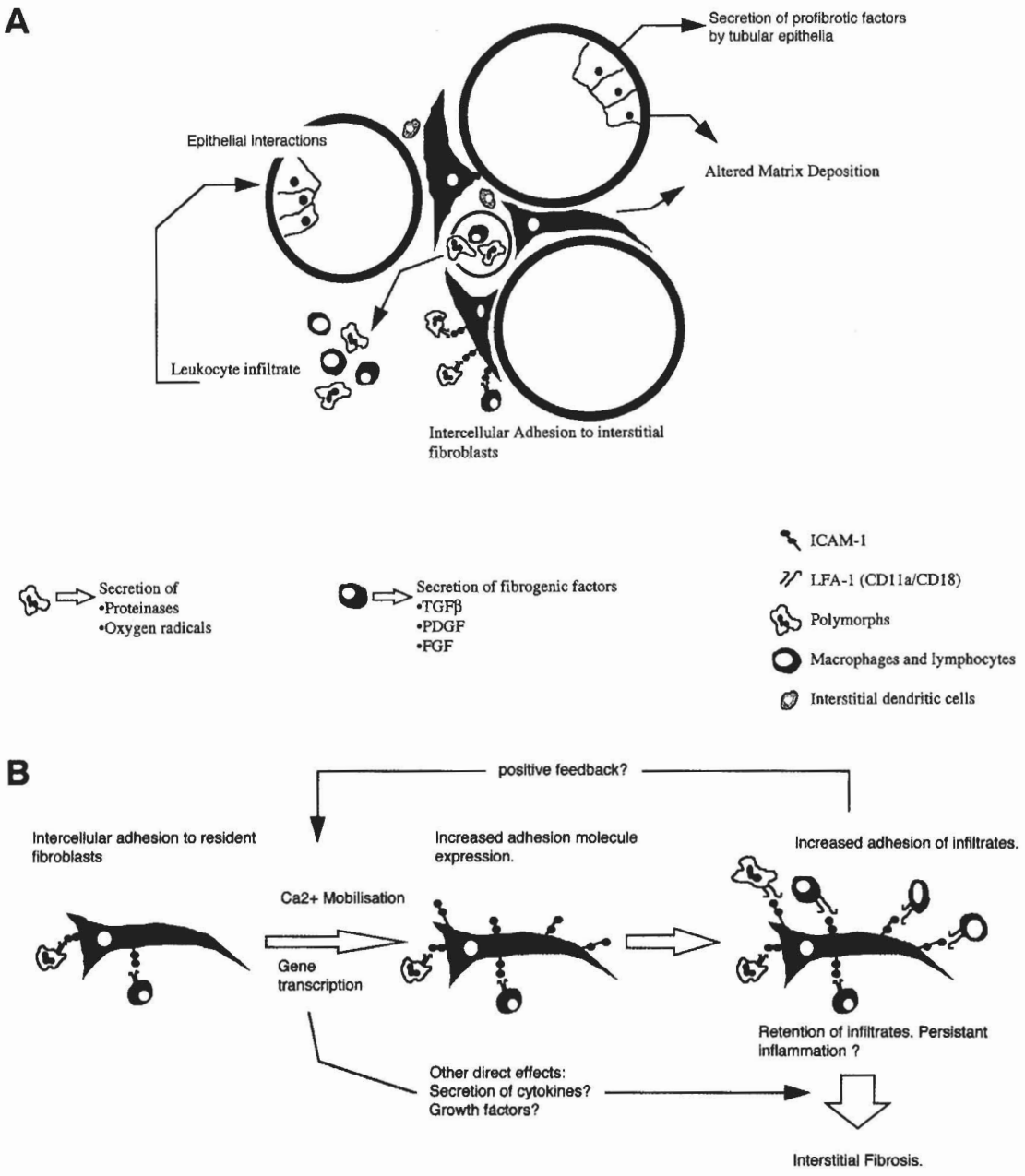


Fig. 5. A. Fibroblast interactions within the inflamed interstitium: The extravasation of leukocytes from peritubular capillaries into the interstitial compartment brings them into close physical apposition with resident fibroblasts. As well as direct cellular adhesion mediated through specific adhesion molecules, the infiltrate may disturb the local environment through the secretion of proteinases, and oxygen radicals leading to damage to resident cells and to the ECM. The degradative products from ECM breakdown may stimulate resident cells. The release of pro-fibrotic factors may also modulate the behaviour of resident cells. **B.** The direct consequence of fibroblast-leukocyte adhesion: ICAM-1 mediated cell activation results from intercellular adhesion, giving rise to the induction of adhesion molecules on the fibroblast surface. This increased adhesiveness in turn promotes further leukocyte binding. Thus a self perpetuating system ensures that leukocytes are retained within the interstitium, where they may promote fibrosis through a variety of mechanisms, such as growth factor secretion. Other direct effects of adhesion on fibroblast behaviour remain to be investigated.

molecule expression (Bombara et al., 1993), yet the involvement of specific adhesion molecules in this effect was not shown. We investigated the effect of ICAM-1 ligation on adhesion molecule expression by fibroblasts. The ligation of ICAM-1 led to the induction of mRNA for ICAM-1 (Fig 4A). Furthermore, we demonstrated that ICAM-1 ligation upregulated the expression of ICAM-1 and VCAM-1 protein (Fig. 4B). Although we do not fully understand this mechanism of ICAM-1 induction, we have demonstrated that blocking cytosolic calcium elevations inhibited this upregulation. Chelating extracellular calcium abolished the cross-linking mediated upregulation of cell surface ICAM-1. Furthermore, the incorporation of a cytosolic calcium buffer (BAPTA) also inhibited cross-linking induced changes in ICAM-1 mRNA.

Adhesion molecule upregulation by this mechanism may provide an important pathway in inflammatory reactions, whereby migrating leukocytes, following extravasation, encounter and adhere to interstitial fibroblasts *via* ICAM-1 or VCAM-1. The subsequent fibroblast activation leading to upregulated adhesion molecule expression, may render the fibroblasts more adhesive. Thus the binding of leukocytes could in turn promote further leukocyte binding, amplifying the retention and/or recruitment of invading leukocytes in the interstitium during nephritis (Fig. 5).

The detailed characterisation of the cortical fibroblast will enable a comprehensive analysis of its responses to different environments to be carried out. Furthermore, since markers for the cortical fibroblast are now available, it will be possible to document the involvement and degree of activation of the fibroblasts at different stages during the progression of human or animal models of disease. Since the cortical fibroblast may be centrally involved in controlling the progression and resolution of injury, greater understanding of the complex factors involved in both the onset of fibrosis and in its resolution, will be of great importance for the treatment of renal diseases.

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