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Polymerase chain reaction and focal contact formation indicate integrin expression in mesangial cells

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Polymerase chain reaction and focal contact formation indicate integrin expression in mesangial cells. Cultured kidney glomerular mesangial cells (MCs) allow the role of extracellular matrix (ECM) and growth factors in glomerular inflammatory disease to be studied. To investigate the potential of MCs to interact with matrix components, the expression of integrin mRNA in cultured MCs was examined by polymerase chain reaction (PCR), by Northern blotting and by immunofluorescence. In addition, the effect of matrix substrates on mRNA expression was assessed by PCR. Northern blots with cDNA probes to integrin α-chains revealed that MCs expressed $\alpha 1$, $\alpha 3$ and $\alpha 5$ integrin mRNA. $\alpha 1$ and $\alpha 3$ were the major messages. No $\alpha 2$, $\alpha 4$ or $\alpha 6$ were detectable. RT-PCR revealed that $\alpha 2$ and $\alpha 6$ were also expressed at low levels. The control cells, HT1080, expressed $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ mRNA, and Rugli expressed $\alpha 1$, $\alpha 3$, and $\alpha 5$, supporting previous studies. Immunocytochemistry confirmed that $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 5\beta 1$ integrins were expressed and that they were concentrated into focal adhesions ($\alpha 1\beta 1$ on type I collagen and laminin; $\alpha 2\beta 1$ on type I collagen; $\alpha 3\beta 1$ on type I collagen, laminin and fibronectin; $\alpha 5\beta 1$ on fibronectin). $\alpha 6\beta 1$ was not detected in focal contacts. Attachment, spreading, and formation of talin and integrin containing focal contacts still occurred when endogenous protein synthesis was blocked with 30 $\mu g \cdot ml^{-1}$ cycloheximide. Variation of substrate did not lead to a rapid degradation of integrin α-chain mRNA as assessed by RT-PCR. These results provide a basis for studying the regulation of interactions between MCs and extracellular matrix mediated by integrins.

Regulated cell interaction with the extracellular matrix occurs in diverse processes of tissue development and remodeling; failure of the regulation characterizes many pathological conditions. Matrix components can influence cell attachment, motion, differentiation and proliferation, and resemble solid phase analogues of the soluble growth factors [1]. In recent years it has become apparent that the integrins are a major class of cell-surface receptors involved in cell-matrix interactions.

The smooth muscle-like cells of the glomerular mesangium are embedded in and interact with a basement membrane-like extracellular matrix. This interaction contributes to the permeability of the glomerulus, both in regulation of fluid flow through the glomerular capillary tuft, and restriction of access for invading leukocytes [2, 3]. The mesangial matrix contains collagen type IV, laminin, fibronectin and various proteoglycans [4, 5]. In the chronic glomerular diseases that lead to glomerulosclerosis, inflammatory processes induce changes in the matrix and the proliferation of the mesangial cells [4–6]. These events contribute to scarring, blocking of the glomerular capillary tuft and kidney failure. Extracellular matrix can modulate cell behavior, however, little is known about mesangial cell (MC) interactions with matrix molecules. $\beta 1$-series integrins often mediate cell-matrix interaction. Integrins are α-β heterodimeric transmembrane receptors whose specificity reflects the α- and β-chains of the complex [7]. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [8–11] bind collagens and laminin [12–14]. $\alpha 3\beta 1$ may bind laminin, collagen type I, fibronectin, entactin, epiligrin and the $\alpha 2\beta 1$ integrin [15–19]. $\alpha 4\beta 1$ [20] and $\alpha 5\beta 1$ [21] bind fibronectin. $\alpha 6\beta 1$ and $\alpha 7\beta 1$ bind laminin [22, 23].

Immunohistochemical studies show that MCs express integrins *in situ* [24–26] and use multiple receptors to attach to the same matrix component [24], but integrin expression, organization and the matrix partners involved have not been fully characterized; there is still debate about exactly which integrins are expressed in MCs and under what circumstances. There have been few attempts to use molecular biological methods to analyze integrins in MCs. The use of antibodies for identifying integrins can be problematical as blocked epitopes, antibody cross reactivity, lack of sensitivity and functional irrelevance may all influence immunocytochemical analysis. In addition, the amount of a given integrin on the cell may be constant, but its function may change following activation [7, 27, 28].

Thus, supporting non-immunologically based data are helpful when probing for integrins. We have studied mRNA transcripts of the integrin α-chains in MCs and integrins in focal adhesions, morphologically well defined structures [29–31]. Neither mRNA expression nor the focal contact distribution of integrins in MCs has previously been examined. We cloned $\beta 1$-series integrin cDNAs from human and rat cells using polymerase chain reaction (RT-PCR), and then employed them as probes in Northern hybridization and in RT-PCR analysis of MCs. In addition, we used RT-PCR to assess whether there was activation or inactivation of transcription of integrin messages in MCs grown on different matrix substrates. Here we describe that $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ integrin chains are transcribed in MCs and are co-opted into focal adhesions. The transcription is not rapidly altered as a short-term response to substrate.

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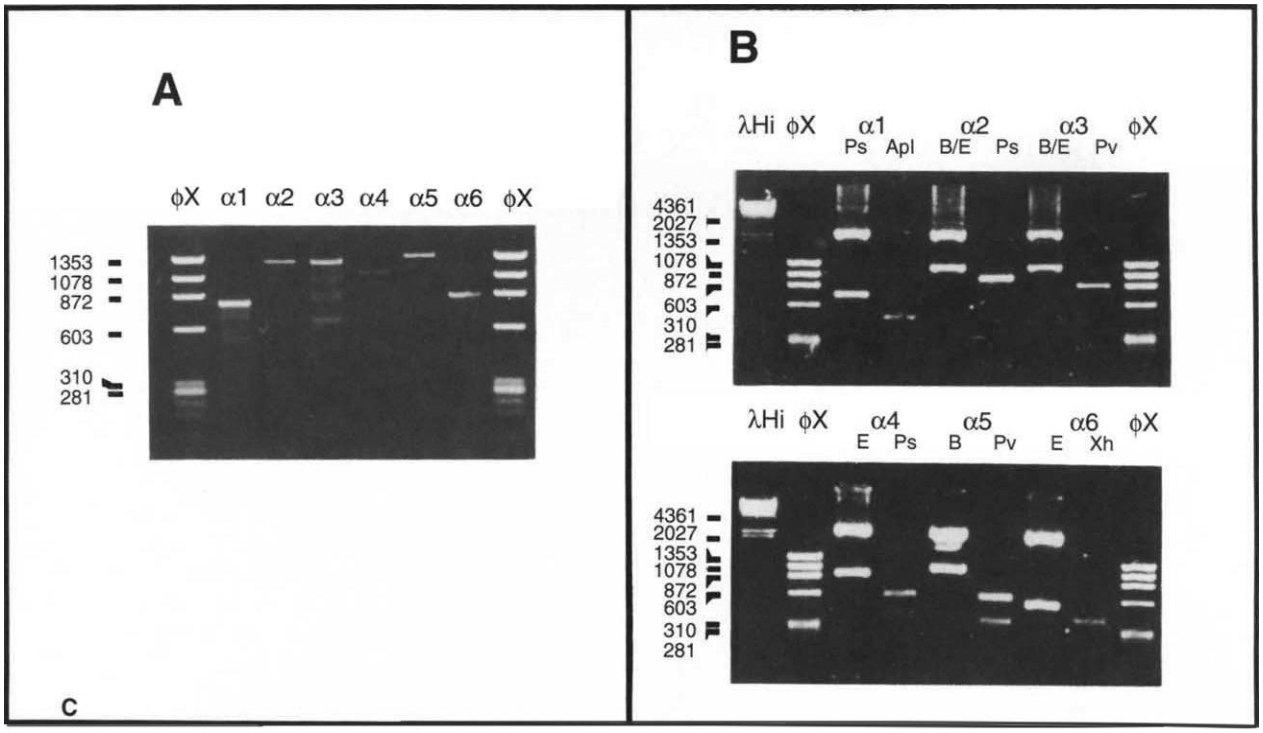

Fig. 1. *Molecular cloning of integrin alpha chains.* (**A**) RT-PCR amplification of integrin mRNA from Rugli and HT1080 cells. Reverse transcription of 5 μg of total RNA, 30 cycle PCR reaction. Ten percent of the reaction volume has been loaded on a 1% agarose gel and the bands visualized with ethidium bromide. ϕX = ϕX174 markers; α1 = α1-integrin primers on Rugli RNA; α2, α3, α4, α5, α6 = α2-α6 integrin primers on HT1080 RNA. Marker sizes in base pairs are shown. (**B**) Cloned integrin insert and partial restriction maps. α1-6 are integrin α-chains from which clones were derived. Enzyme digests of clones alternate with digests of their inserts. PstI fragment α1 ex pUC19 (pRI1.1); BamHI-EcoRI fragment α2 ex pUC18 (pHI2.1); BamHI-EcoRI fragment α3 ex pUC18 (pHI3.1); fragment Gkα5 EcoRI fragment α4 ex pUC18 (pHI4.1). BamHI-EcoRI fragment α5 ex pUC18 (pHI5.1). EcoRI fragment α6 ex pUC18 (pHI6.1). ϕX = ϕX174 markers: λH = λ-HindIII markers. ApI = ApAI, E = EcoRI, B = BamHI, Ps = PstI, Pv = PvuI, X = XhoI. (**C**) Partial restriction maps of cloned inserts.

Methods

Unless otherwise stated, chemicals were of analytical grade from Merck (Darmstadt) and biochemicals were from Sigma. Tissue-culture-quality water was used throughout. Reagents for polymerase chain reaction (PCR) and molecular cloning were from Boehringer (Mannheim). Falcon tissue culture plastic was from Becton-Dickenson. Tissue culture media and sera were from Gibco-BRL.

Cell culture

Cell culture of HT1080 (human fibrosarcoma), Rugli (rat glioblastoma) and B16 (mouse melanoma) cells have been described elsewhere [11, 23, 32]. HT1080 express VLA2,3,5 and 6. Rugli express the rat homologues of VLA-1,3,5 and $\alpha 7\beta 1$. B16 express the mouse homologues of VLA 2,5 and 6.

Preparation of MCs. Standard techniques were used [33, 34]. The cortex of rat kidneys was cut into fragments and pressed through stainless steel sieves. Glomeruli retained between 105 μm and 75 μm sieves were gathered, washed and plated in D20 (DMEM, 20% heat-inactivated FCS, 2 mM glutamate, 5 ng/ml insulin, 100 U/ml penicillin, 1000 μg/ml streptomycin) at 3×10^4 glomeruli per 75 cm^{-2} flask. MCs grew out from the glomeruli within three to six weeks. After three to four passages, the population was shifted to D10 (D20, but 10% in FCS). Human

MC cultures from kidneys not suitable for transplantation were prepared in a similar manner, but were maintained in R10 [RPMI in place of DMEM, plus ITS (Boehringer; 10 $\mu g \cdot ml^{-1}$) and HEPES (15 mM; pH 7.3)]. MCs were withdrawn from the cell cycle by incubation for 120 hours in D0.5 (D20, but 0.5% FCS). Rat and human MCs showed positive staining for Thy1.1, vascular smooth muscle cell myosin, actin and desmin, and had characteristic morphology [33, 34]. The cultures showed uniform marker expression and no expression of MAC-1 and factor VIII, which are, respectively, markers for macrophages and endothelial cells [34].

Molecular biological methods

Standard methods were used, where not otherwise detailed [35].

Reverse transcription-polymerase chain reaction. PCR primers with minimal secondary structure or cross homology were selected from integrin alpha-chain sequences (Rat α1 [36], and human α2 [37], α3 [38], α4 [39], α5 [40] and α6 [41]) and synthesized with terminal recognition sites for the restriction endonucleases BamHI, EcoRI, or PstI. The primers used were: Rat α1-up 5'CTACAAGATGGAGGATGGG 3' (bp.1817-1835 + PstI), Rat α1-down 5'GCCTCAGTGAATCAAGGG 3'(bp 2590-2607 + PstI); Hu α2-up 5'AGCTGGATCCACTCTGCTTCAGTGCAAAGTTCAGA 3'(bp.2082-2106 + BamHI), Hu α2-down 5'GTCAGAATTCAAAGTCCCGTTCCAAATTCTGGTAG 3' (bp. 3275-3299 + EcoRI); Hu α3-up 5'ATGCGAATTCTGTGGTCAGGGTCAGAAGACG 3' (bp. 516-535 + EcoRI), Hu α3-down 5' TCATGGATCCGGAGAAGAAGCCGTGGAAGACAGCG 3'(bp. 1681-1705 + BamHI); Hu α4-up 5'GATCGGATCCGACGTGATTACAGGAAGCATACAGG 3'(bp.1666-1691 + BamHI), Hu α4-down 5' TGACGAATTCGCAGTACAATAGCCTCTTATCAGTC 3' (bp. 2688-2713 + EcoRI) [39]; Hu α5-up 5'TCAGGGATCCAACTTCAGCTGGACTGGCAGAAGCA 3'(bp.1657-1675 + BamHI) Hu α5-down 5'GATCGAATTCGGGCATCTTCAGGGCTTTGTACACA 3'(bp.2900-2925 + EcoRI); Hu α6-up 5'TCAGGGATCCGTGTTGCCAACCAGAATGGCTCGC 3'(bp.2323-2346 + BamHI) Hu α6-down 5'GATCGAATTCCAGTCACTCGAACCTGAGTGCCTGC 3'(bp.3123-3147 + EcoRI).

For the validation of the PCR primers, total RNA (1 to 5 μg) [42] from HT1080 or Rugli cells was transcribed (1 hr × 37°C) with MMLV reverse transcriptase (200 U; Superscript, Stratagene) using integrin downstream primer (30 pmol) in a volume of 20 μl (75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris Cl; pH 7.3). A quarter of the reverse transcription mix was used for PCR (30 cycles; 35 seconds × 95°C, 30 seconds × 50°C, 150 seconds × 72°C) by adding dNTPs (20 nmol), up and downstream primers (to 50 pmol) and Taq-polymerase (2.5 U; Stratagene) in 100 μl (50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 mM Tris-Cl; pH 8.8). Amplified fragments were analyzed by electrophoresis on agarose gels.

Cloning. cDNAs amplified by PCR were purified, cleaved with restriction endonucleases (to generate PstI fragments for rat-α1 and BamHI-EcoRI fragments for α2, α3, α4, α5 and α6), purified on agarose gels, and cloned into pUC18 or pUC19. Inserts were analyzed by restriction mapping and by sequencing using the dideoxy method, which confirmed their identity with the published sequences [36–41].

Northern hybridization analysis. Cloned integrin cDNA fragments were labeled with P^{32}-ATP (Amersham) by random priming (Gibco) to $\approx 1 \times 10^8$ dpm $\cdot \mu g^{-1}$. Total RNA (30 μg) was resolved on agarose/formaldehyde gels, transferred to nylon membranes (Hybond-N; Amersham) by capillary wicking, and fixed by baking (2 hr × 80°C). After pre-hybridization (2 hr × 38°C; 50% formamide, 5 × SSC, 0.5 × Denhardts solution, 200 $\mu g \cdot ml^{-1}$ herring sperm DNA, 1% SDS, 50 mM phosphate buffer; pH 6.5) the membranes were hybridized with the labeled cDNA probes (16 hr × 38°C). The membranes were washed (38°C: 2 × 15 min, 2 × SSC, 0.1% SDS; 2 × 30 min, 0.2 × SSC, 0.1% SDS) and exposed to X-ray film (Kodak, XAR-5) at −80°C. Filters were rehybridized following stripping in boiling SDS (0.1%; 3 ×1 min). Hybridization with gamma-actin cDNA probes revealed comparable quantities of gamma-actin message in each lane. The size of the hybridizing species was estimated from the migration of the 28S and 18S rRNA [35].

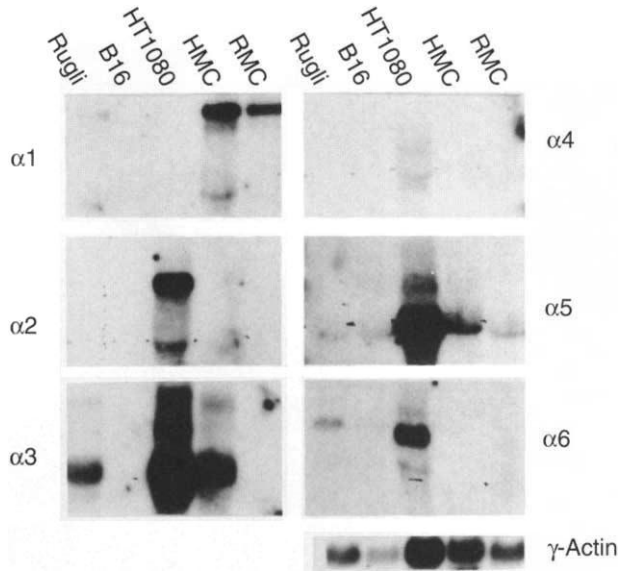

Fig. 2. *Northern hybridization analysis of integrin expression in MCs.* Total RNA (30 μg) was run on 1% agarose-formaldehyde gels, transferred to nylon membranes and serially probed with radiolabeled integrin inserts shown in Figure 1B. HMC = human MCs, RMC = rat MCs. All films were exposed for 72 hours. The nature of the probes used is shown (α1-α6).

Effect of substrate on integrin gene expression

Human vitronectin [43], fibronectin [44], murine EHS-tumor laminin [45] and human collagen type I (gift of Dr. K. von der Mark, Max-Planck, Erlangen, Germany) were >95% pure by SDS-PAGE. The proteins were dissolved in PBS (20 $\mu g \cdot ml^{-1}$) and coated (1 hr × 37°C) on 6-well tissue culture plates (Falcon). After blocking of free protein binding sites (2% heat denatured BSA in PBS; 2 hr × 4°C), quiescent MCs were added (5×10^4 cells/well) in A-buffer (DMEM, 0.5% BSA, HEPES, 20 mM; pH 7.3). At six hours the cells were harvested, and total cellular RNA was extracted [42]. Integrin cDNAs were amplified using RT-PCR ($\approx 10^4$ cells $\approx$10 ng RNA per PCR) and the products were resolved by agarose gel electrophoresis. Parallel amplifications with RNA from HT1080 and Rugli cells provided

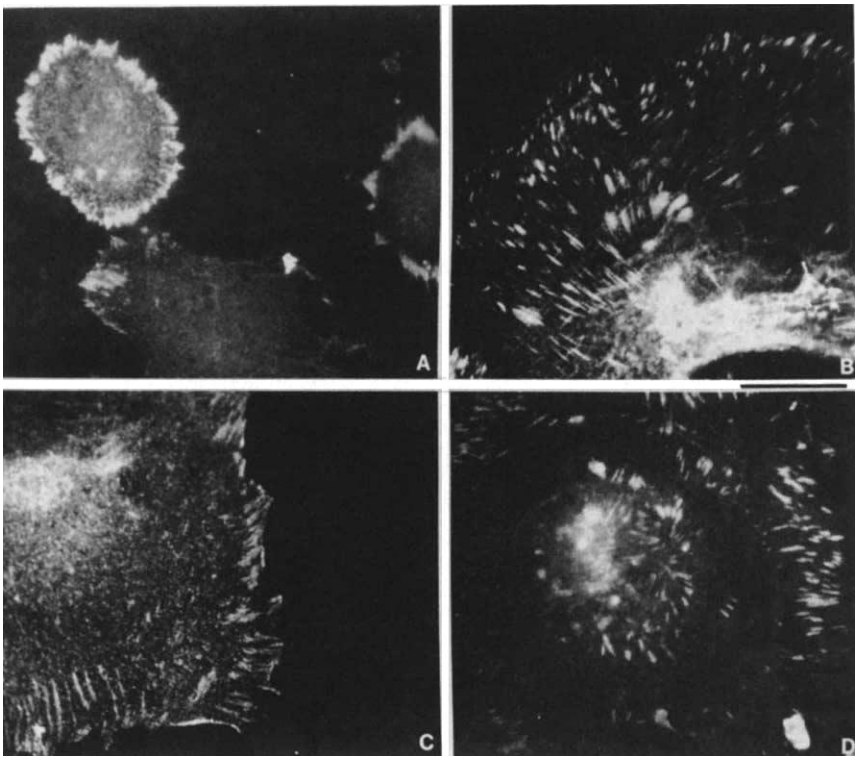

Fig. 3. *Immunofluorescent labeling of integrins in MCs.* MCs were plated on (**A**, **B**) collagen or (**C**, **D**) fibronectin for 4 hours before fixation and labeling for (**A**) $\alpha 1$, (**B**) $\alpha 2$, (**C**) $\alpha 3$, (**D**) $\alpha 5$ integrin chains. Rat MCs are shown in A and human MCs in B through D. Scale bar = 10 μM.

markers for the amplified products, which after Southern transfer were confirmed by hybridization with the subcloned integrin cDNAs.

Antibodies

Murine primary antibodies against human integrin alpha chains P1E6 ($\alpha 2$), P1B5 ($\alpha 3$), and P1D6 ($\alpha 5$) [15] and rabbit anti-fibronectin were from Telios (La Jolla, California, USA). Other antibodies were gifts of our colleagues: 7C7C7 (mouse anti-human $\alpha 4$; Dr. J. Cassiman and S. Vekemans, Leuven), GOH3 (rat anti-mouse $\alpha 6$ [46]), 3A3 (mouse anti-rat $\alpha 1$ [9]), AIIB2 mouse anti-$\beta 1$ integrin [10] and rabbit anti-talin [47]. Second layer fluorescent labeled antibodies were Texas-red donkey-anti-mouse (Amersham) and Texas-red goat-anti-rat (Dianova). Rabbit anti-collagen-I was the gift of Dr. K. von der Mark (Erlangen) and rabbit anti-laminin was produced by immunization with EHS-laminin. TRITC-phalloidin was from Molecular Probes (Oregon)

Immunofluorescence

Immunofluorescence microscopy will be described elsewhere (**Note added in proof**). Briefly, MCs were plated for four hours in A-buffer on multichamber slides (Nunc) coated with matrix protein (20 $\mu g \cdot ml^{-1}$ in PBS). After washing and fixation with paraformaldehyde (3% wt/vol in PBS: 10 min × 0°C), the cells were permeabilized (Triton X-100; 1% (wt/vol) in PBS; 10 min × 20°C), and incubated with primary antibody followed by fluorescently labelled secondary antibody before mounting in PVA (Moviol 4-88, pH 8.6; Hoechst). The cultures were examined under a Leitz Axioplan microscope equipped for epifluorescence microscopy, and photographed at 3200 ASA on TMAX film (Eastman-Kodak). Staining patterns qualitatively as those described here developed as soon as the cells could be fixed (0.5 to 1 hr) but were most extensive by four hours.

Cycloheximide treatment

Three hours before plating for immunofluorescence, cycloheximide to a final concentration of 30 $\mu g \cdot ml^{-1}$ was added to MC cultures. Subsequently, it was present in all solutions that came into contact with the cells until they were fixed for immunostaining. Endogenously produced matrix components laminin, fibronectin and collagen type I were no longer detectable after this procedure (**Note added in proof**).

Results

Human and rat MCs transcribe integrin message

RT-PCR was used to amplify mRNA transcripts from Rugli ($\alpha 1$) and HT1080 cells ($\alpha 2$-$\alpha 6$). Integrin fragments of the predicted sizes ($\alpha 1$, 790 bp; $\alpha 2$, 1237 bp; $\alpha 3$, 1209 bp; $\alpha 4$, 1066 bp; $\alpha 5$, 1287 bp; $\alpha 6$, 844 bp) could be amplified (Fig. 1a). When cloned into pUC18 and pUC19 to generate unique cDNA probes, the restriction maps (Fig. 1 b and c) and partial 2 to 300 bp sequences matched published integrin data (AP, HF and SLG, unpublished observations).

The cloned integrin cDNAs were used to probe MCs using Northern hybridization (Fig. 2). Integrin $\alpha 1$ cDNA probes yielded signals from both human and rat MCs, and from Rugli. A strong signal was seen at 11.5 ± 2 kb and a weak signal at 5.5 kb. Integrin $\alpha 2$ probes detected a signal only from HT1080 cells, at 9.0 ± 2 kb. $\alpha 3$ probes detected signals in human MCs and in

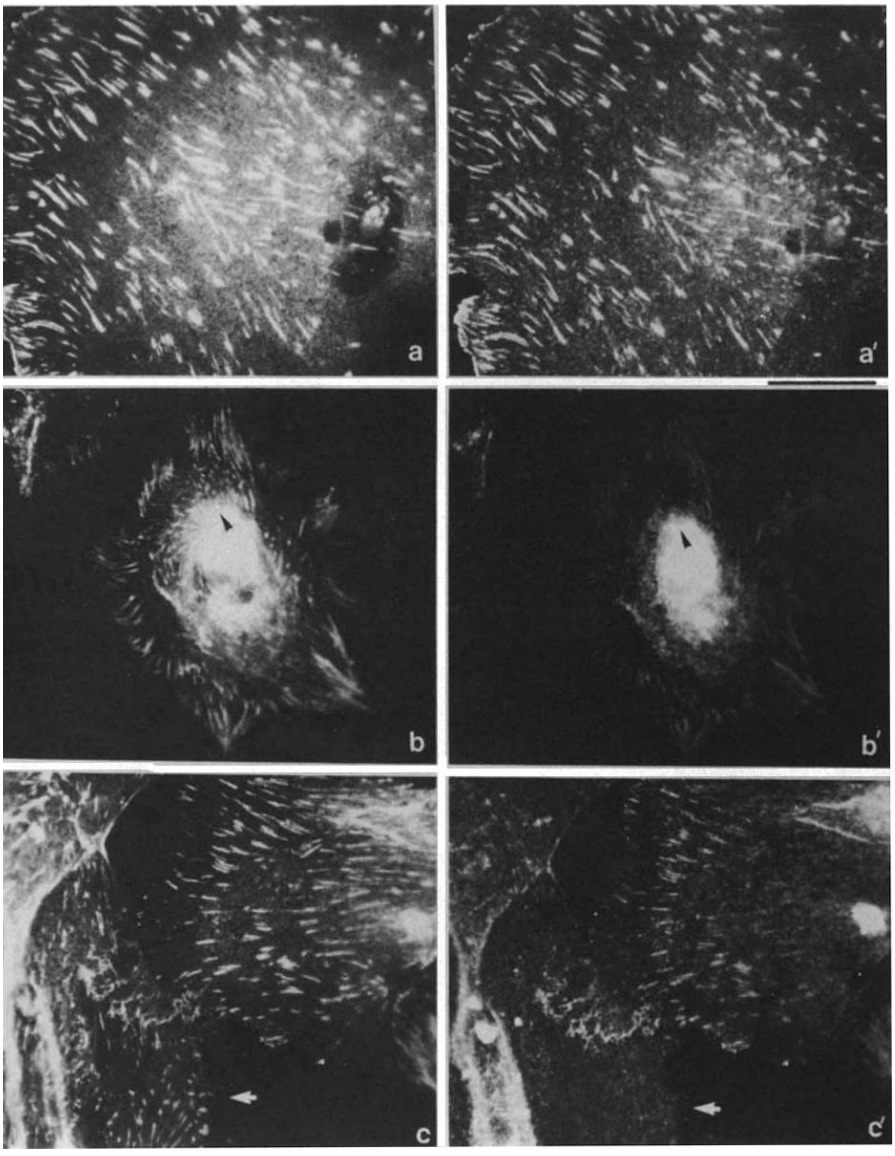

Fig. 4. *Colocalisation of talin and integrins in MCs.* Human MCs were plated on fibronectin and fixed and double labeled at 4 hours for talin (**a,b,c**) or the integrins $\beta 1$ (**a'**), $\alpha 3$ (**b'**) or $\alpha 5$ (**c'**). Arrowheads in (b,b') and (c,c') indicate heterogeneous labeling of focal contacts. Scale bar = 10 μM.

Rugli and HT1080, with a major signal at ≈ 5.5 kb and a minor signal at ≈ 12 kb. Rat MCs and B16 expressed no $\alpha 3$. Only in HT1080 cells did the $\alpha 4$ probe detected a weak signal, at 6.5 kb. The $\alpha 5$ probe gave a signal at ≈ 5.0 kb, it was prominent in MCs, strong in HT1080 cells and weak in Rugli and B16. $\alpha 6$ probes gave signals only in HT1080, and weakly in B16 and Rugli at ≈ 7 kb. Weak bands in the HT1080 lane were visible at ≈ 12 kb and ≈ 4.5 kb. Minor bands may represent mRNA processing variants. The major bands migrated (within the measurement errors) at similar rates to the integrin mRNAs described in the literature [36–41].

MCs have integrins in focal contacts on appropriate substrates

MCs formed focal contacts on extracellular matrix (Fig. 3). $\alpha 1\beta 1$-containing focal contacts (FCs) formed in rat MCs on collagen type I and laminin but not on fibronectin or vitronectin. $\alpha 2\beta 1$-containing FCs formed in human MCs on collagen, but not on other substrates. On fibronectin, but not on collagen and laminin, they formed $\alpha 5\beta 1$-containing FCs. Surprisingly, $\alpha 6\beta 1$ was not in FCs on any substrate. But $\alpha 3\beta 1$ was in FCs on collagen type I, laminin and fibronectin (Fig. 3). Double labeling for the $\beta 1$ integrin and talin showed precise co-distribution in FCs (Fig. 4). In control cells, HT1080 had $\alpha 2\beta 1$ in FCs on collagen, $\alpha 5\beta 1$ in FCs on fibronectin, and $\alpha 6\beta 1$ in FCs on laminin, Rugli had $\alpha 1\beta 1$ in FCs on collagen and laminin. $\alpha 3\beta 1$ was not in FCs.

Thus, $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ integrin chains were in adhesive complexes in MCs. The majority (>80%) of the MCs localized integrins into talin-positive FCs as predicted from the integrin specificities described by the literature. These data will be discussed in detail elsewhere (**Note added in proof**).

MC cells can synthesize extracellular matrix. To test whether focal contact formation was dependent on endogenously pro-

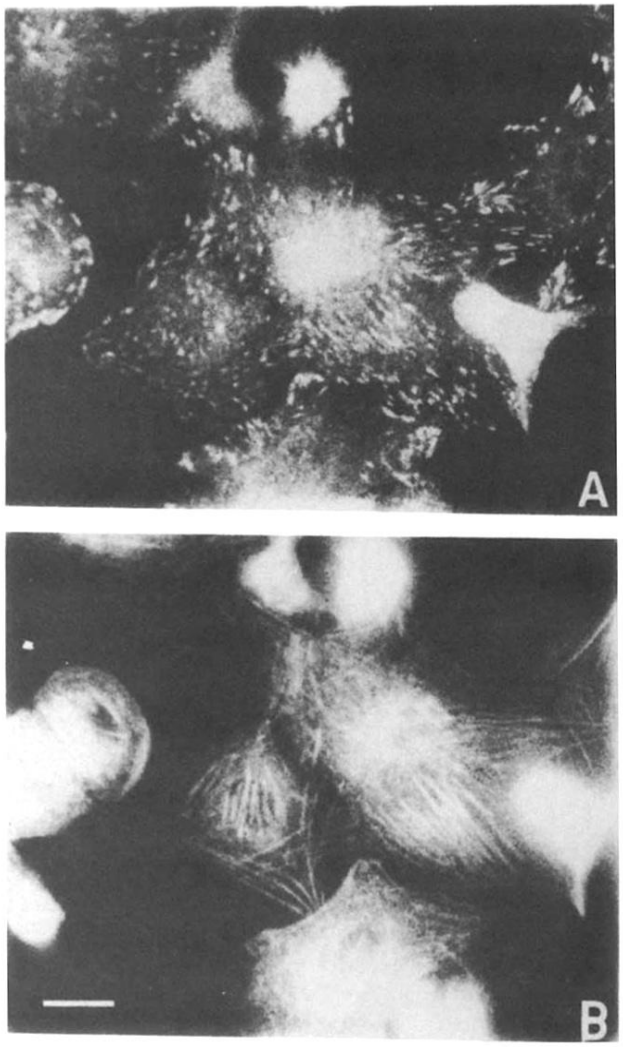

Fig. 5. *Integrin and cytoskeletal organization proceeds in the absence of endogenous protein synthesis.* MCs were preincubated for 3 hours with 30 $\mu g \cdot ml^{-1}$ cycloheximide before trypsinization and 4 hours attachment to collagen I in the continuous presence of cycloheximide, before being fixed and double labeled for (**A**) $\alpha 1\beta 1$ integrin and (**B**) actin (phalloidin stain). Note the spread cells, the normal actin cytoskeleton and the $\alpha 1\beta 1$ integrin in focal contacts. Scale bar = 10 μM.

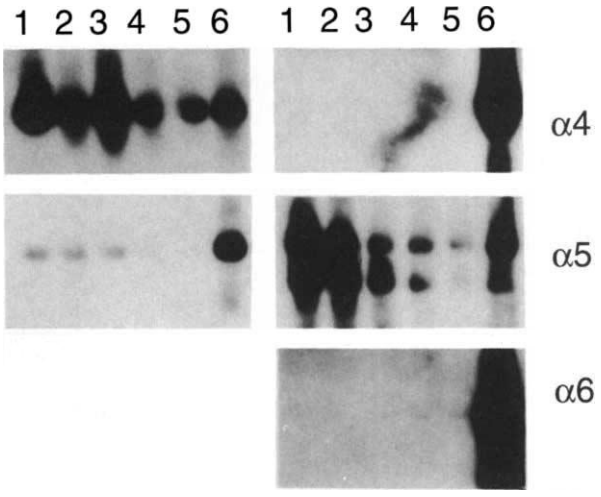

Fig. 6. *PCR analysis of integrin expression in response to matrix substrates.* RNA from $\approx 8 \times 10^4$ MCs or control cells (Rugli for rat MCs; HT1080 for human MCs) plated for 6 hours on the substrates shown was amplified by RT-PCR using integrin specific-primers, the reaction products were resolved on 1.5% agarose gels, transferred to nitrocellulose and probed with labeled integrin inserts shown in Figure 1. Lanes are (1) MCs on serum; (2) MCs on fibronectin; (3) MCs on vitronectin; (4) MCs on laminin; (5) MCs on collagen type I; (6) control cells on serum. Rat MCs probed for $\alpha 1$ were at a 0.5 hour exposure. Human MCs probed for $\alpha 2$ were an 8 hour exposure, for $\alpha 4$ a 16 hour exposure, for $\alpha 5$ a 16 hour exposure, and for $\alpha 6$ were a 7 hour exposure.

duced proteins, protein synthesis was blocked by cycloheximide, and attachment and focal contact formation was studied. Cell attachment and spreading, the construction of the actin-talin cytoskeleton and the concentration of integrins into focal contacts in a substrate-dependent manner were not affected by cycloheximide (Fig. 5). Endogenous matrix production (judged by immunofluorescence) was abolished (**Note added in proof**).

Growth on a particular matrix substrate does not cause rapid switching of integrin expression in MCs

To increase the sensitivity of the mRNA analysis and to assess whether substrate substantially affected integrin expression, quiescent MCs were plated onto matrix molecules and integrin mRNA was amplified using PCR. The PCR products were probed with the cloned integrin cDNAs to confirm the identity of the amplified message (Fig. 6). Rat MCs expressed $\alpha 1$ by RT-PCR. Human MC expressed very low levels of $\alpha 2$ message even after PCR amplification compared to HT1080. A signal was seen using $\alpha 3$ primers in HMCs and HT1080, without further probing (data not shown). There was no $\alpha 4$ signal from MCs, while HT1080 gave a strong signal. $\alpha 5$ gave signals in HT1080 and MCs on all substrates. The second signal may represent a minor splice variant form of $\alpha 5$ mRNA. Human MC expressed very low levels of $\alpha 6$ message, even after PCR amplification, compared to HT1080 on all substrates.

In summary, the qualitative PCR analysis revealed integrin $\alpha 1,2,3,5$ and 6 mRNAs in MCs. Given the limitations of the method, there was no evidence that the substrate caused a rapid shut down or activation of integrin gene transcription.

Discussion

Integrins can mediate cell interaction with the extracellular matrix [7]. The regulation of integrin function may play a role in the disease process, and this is of interest in glomerular sclerosis and in other diseases where alteration of cell-matrix interactions occurs [4–6]. A knowledge of integrin activity and expression may suggest therapeutic or preventative approaches. Changes in mesangial cells and matrix are a hallmark of progressive glomerular disease [4–6, 34], but little is known about the regulation of integrin expression and activity in the MC system. The matrix of MCs has been extensively investigated [24–26], but the receptors they use to recognize it has not. Integrins in tissue sections of kidney have been examined, but only one study has considered MCs in culture [24]. Yet, there is disagreement about which integrins are in MCs. Here, we have used PCR amplification of integrin mRNA and immunofluores-

cence microscopy to study integrins in cultured MCs. We find that (a) MCs express mRNA for integrins α1,2,3,5 and 6 mRNA; (b) that α1,2,3 and 5 localize to focal contacts independently of endogenous protein synthesis; and (c) there is no rapid on/off switching of integrin mRNA in response to the matrix.

The level of integrin α-chain mRNA reflects the potential level of $\alpha\beta$-complex at the cell surface [48, 49], while the presence of integrins in focal contacts accompanies their usage for attachment. We generated specific PCR primers and cDNA probes for the integrin α-chains. The antibodies used have been characterized elsewhere [11, 17, 23, 50, 51]. In Northern analysis we detected α1, α3 and α5, and by PCR α2 and α6 in MCs. Immunofluorescence confirmed that $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha5\beta1$ were present in focal contacts. $\alpha6\beta1$ could not be seen on the cell surface. The distribution pattern of integrins in focal contacts was unaffected by blocking endogenous protein synthesis, suggesting that the cells were responding to exogenous rather than endogenous (that is, secreted) proteins. Indeed, it was remarkable how little effect on attachment, spreading and cytoskeletal organization this long blockade with cycloheximide had on cells. Similar observations on the lack of effect of cycloheximide on spreading and focal contact formation have recently been made [52].

α1

MCs synthesize α1 [24–26], but in human MCs none was found by immunostaining [24]. Here we find α1 mRNA in human MCs and rat MCs, and in rat MC focal contacts. $\alpha1\beta1$, a potential collagen/laminin receptor, is present in cultured MCs. The discrepancy in the literature may have arisen because $\alpha1\beta1$ is only clearly visible in focal contacts, as when MCs are on laminin or especially collagen, and not on vitronectin or fibronectin (the substrates that form when cells are plated in serum-containing medium). $\alpha1\beta1$ is characteristic of smooth muscle cells *in situ* [53] and MCs have several features of smooth muscle.

α2

$\alpha2\beta1$ is in the mesangium on kidney sections [26], but it was not found in cultured MCs [24]. On MCs we find very low levels of α2 mRNA, while $\alpha2\beta1$ protein is clearly found in focal contacts on collagen. Although there is no strict relationship between the level of a particular mRNA and the resulting protein expression, the reason for the very low levels of α2 mRNA are far from clear. Perhaps α2 mRNA is very unstable, although there have been no reports in the literature that would support this. However, the result is probably correct. The MC population is relatively clean judged by the homogenous staining with MC-specific markers (such as desmin) and for $\alpha2\beta1$ (that is, about 80% positive cells), and the defining anti-α2 antibody (P1E6) is highly specific. We have shown elsewhere that the development of α2-containing focal contacts is highly collagen dependent (**Note added in proof**) and that the defining antibody only precipitates $\alpha2\beta1$ from cells [23]. In MCs the previous failure to find $\alpha2\beta1$ may be due to the cells not having been on collagen, only there is it highly concentrated and so visible in focal contacts (as suggested for α1 above).

α3

There are two reports of $\alpha3\beta1$ in MCs in tissue [24, 26], and one in culture [24], while one group found little $\alpha3\beta1$ in tissue [25]. We found that α3 mRNA was expressed in human MCs and $\alpha3\beta1$ localized to focal contacts.

α4

α4 mRNA was not detected in MCs which agrees with previous reports.

α5

There is one report of significant quantities of $\alpha5\beta1$ in glomerular tissue and as a fibronectin receptor in human MCs [24]. We found α5 is transcribed in human and rat MCs and it localizes in focal contacts on fibronectin.

α6

RT-PCR but not Northern analysis or immunofluorescence of MCs revealed α6. The very low levels of α6 transcript therefore may represent non-regulated breakthrough transcripts.

It is of interest to establish whether the substrate regulates the matrix receptors expressed at the cell surface. As integrin proteins are long-lived [48], we investigated whether mRNA levels were regulated in response to substrate. There was no case where the message for a particular integrin was unequivocally shut off. We did not establish a quantitative PCR, which might have been capable of detecting variations in mRNA expression, but we could amplify five different integrins from RNA derived from 8×10^4 cells, and derived clear signals from about 10 ng total RNA. In the future it will be highly interesting to investigate whether integrin expression patterns vary when mesangial cells are allowed to develop into three-dimensional hillock cultures [6]. In numerous cellular systems, the switch from two dimensional to three dimensional growth, and subsequent differentiation has a drastic influence on gene expression.

In summary, we combined mRNA and immunofluorescence analysis to investigate integrin α-chains in cultured MCs. α-chains α1, α2, α3 and α5 detected in Northern hybridization and PCR were found by immunofluorescence in focal adhesions, suggesting that they may be involved in MC attachment. Manipulation of integrin-MC interactions may suggest how they are regulated and function and may lead to the development of new diagnostic or therapeutic approaches in chronic inflammatory disease of the glomeruli.

Note added in proof

Grenz, Carbonetto and Goodman; *J Cell Sci* (in press).

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