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Formation of Extracellular Matrix by Cultured Rat Mesangial Cells

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Formation of extracellular matrix (ECM) by mesangial cells (MCs) contributes to progressive glomerulosclerosis. The authors investigated the production and distribution of ECM constituents by cultured rat MCs, using immunocytochemistry and immunoelectron microscopy. Staining for all ECM constituents increased after serum feeding. Localization was strictly intracellular until confluency, when extracellular deposition of collagen IV and laminin appeared, followed by fibronectin and collagen III. In parallel, the intracellular staining for these proteins diminished markedly. Neither extracellular deposition nor intracellular loss was observed for collagen I and thrombospondin. On surfaces coated with collagen IV or laminin, extracellular deposition of ECM constituents clearly preceded confluency. These results indicate that synthesis of ECM constituents parallels MC growth, and that extracellular deposition of ECM occurs at cell–cell contact. Collagen IV or laminin secreted by MCs in the substratum accelerates production and facilitates secretion of other ECM constituents in an autocrine fashion. (Am J Pathol 1989, 134: 843–855)

The severity of glomerular disease is determined, in part, by the degree of hypercellularity due to intrinsic glomerular cell proliferation and cell infiltration and by the excess production of extracellular matrix (ECM) components. Accumulation of ECM of mesangial origin appears to be especially important for the progression of glomerular sclerosis after various types of glomerular injury, as seen in glomerulonephritis and diabetic nephropathy.[1,2] Mesangial cells (MCs), which are contractile, smooth musclelike cells located in the intercapillary space of the glomerular tuft, have been reported to produce several ECM constituents, such as collagen (Col) types I, III, and IV,[3,4] fibronectin (FN),[4–7] laminin (LM),[8,9] and thrombospondin (TSP),[10,11] as well as various proteoglycans.[12] The factors that regulate ECM production by MCs are at present poorly understood.

In this study, we investigated the formation of several ECM constituents by cultured rat MCs, using immunoperoxidase cytochemistry and immunoelectron microscopy. In particular, we studied the time course of intracellular presence and extracellular deposition of ECM constituents. Furthermore, we examined the relationship of the production and distribution of individual ECM proteins to the proliferative activity of MCs, cell density, and the nature of the underlying substratum.

Materials and Methods

Mesangial Cells

Kidneys were excised from male Sprague-Dawley rats, weighing 175 to 200 g, and placed in sterile Hanks' balanced salt solution (HBSS). The renal cortices were minced and pressed onto sterile stainless sieve of #140, the pore of which is 106 μ in size, and passed through serial sterile sieves of various pore size as described previously.[5,13] On the sieve #200 (pore size, 75 μ), glomeruli were retained almost exclusively (less than 5% tubular fragments). Glomeruli on the sieve were collected, rinsed with HBSS, and centrifuged. The pellet of rinsed glomeruli was resuspended in HBSS containing 750 μg/ml collagenase type IV (Sigma Chemical Co., St. Louis, MO), incubated for 30 minutes at 37 C with gentle agitation, and centrifuged at 1000 rpm for 10 minutes. With this procedure, most epithelial cells were removed and the pellet contained glomerular cores consisting of endothelial and mesangial cells. The pellets of glomerular cores were washed with HBSS and resuspended in complete medium, containing Dulbecco's Modified Eagle's Medium

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(DMEM, GIBCO, Grand Island, NY) culture medium with 20% heat-inactivated fetal calf serum (FCS, Gibco), supplemented with penicillin 100 U/ml (Sigma), streptomycin 100 μg/ml (Sigma), and bovine insulin 5 μg/ml (Sigma). Glomerular cores suspended in complete medium were then plated on 75 sq cm Falcon tissue culture flasks (Becton Dickinson Labware, Lincoln Park, NJ) and cultured at 37 C in humidified 5% CO_2 atmosphere, and fed with complete medium every 3 to 4 days. After 4 to 5 days, two types of cellular outgrowth were observed, as reported previously;[5,13,14] one had typical MC morphology with straplike growth in interwoven bundles, and the other showed epithelial cell morphology with a polygonal, cobble stonelike outgrowth. By 2 to 3 weeks after plating, the epithelial cells disappeared, whereas the MC cell type remained and grew to confluency. These cells, considered and characterized as MCs,[5,13,14] were subcultured onto other plastic flasks and fed twice weekly.

For the experiments, the MCs between the fourth and eighth passages were used. MCs that were confluent in a flask were washed twice with phosphate-buffered saline, pH 7.4, (PBS) with 1 mM EGTA (Sigma), and were incubated in 5 ml 0.01% trypsin-EDTA (Sigma) at 37 C for 3 to 5 minutes. MCs were detached from the surface of the flask by repeated knocking. Ten milliliters of warmed complete medium was promptly poured into the flask to stop the trypsin reaction. These procedures yielded a single-cell suspension of MCs. Cell density of the MC suspension were counted by use of hemocytometer (American Optical, Buffalo, NY).

Plating MCs onto Culture Chamber Slides

For immunoperoxidase staining and immunoelectron microscopy, we used tissue culture chamber glass slides (Nunc Inc., Naperville, IL), which have eight chambers separated by rubber seams and plastic upper house. The bottom surface of each chamber measures approximately 0.79 sq cm and the approximate volume of a chamber is 500 μl. MCs were plated onto the chamber slides at cell concentrations of 250, 500, 1000, 2000, or 4000 cells per chamber suspended in 400 μl complete medium. To halt active growth of MCs,[15,16] 1 or 2 days after plating the outgrowths were serum-starved with medium containing 0.5% instead of 20% FCS for 4 days. The cells were then re-fed with complete medium every 2 days. MC outgrowths were carefully observed by phase contrast microscopy (Olympus) twice daily, and the time of confluency of each culture was recorded.

Some of chamber slides with confluent MC outgrowths were re-fed with the complete medium containing 50 μg/ml ascorbic acid to examine its effect on ECM production and distribution. A preliminary study showed that ascorbic acid was cytotoxic to subconfluent MCs, probably because of a mechanism similar to that reported with cultured fibroblast,[17] where ascorbic acid cytotoxicity at low cell density was attributed to hydrogen peroxide formation.

To evaluate the effect of one ECM constituent on MC production of other ECM constituents, chamber slides were coated with human FN (Sigma), LM derived from murine Engelbreth-Holm-Swarm (EHS) sarcoma, Col I derived from rat tail, Col III derived from human placenta, or Col IV derived from murine EHS sarcoma. These proteins were provided by Dr. J. A. Madri (Yale University School of Medicine).[18,19,20,21] To achieve ECM coating, chamber slides were incubated with 250 μl solution of 10 μg/ml FN, LM, Col I, or Col III, or Col IV dissolved in 50 mM sodium carbonate-bicarbonate buffer, pH 9.6, for 60 minutes at room temperature (22 C), then washed three times with HBSS before plating 1000 cells per chamber. As a control, 1% of denatured bovine serum albumin (BSA) in the same buffer (heated at 80 C for 3 minutes) was used for coating the chamber slides. After plating on coated slides, the cells were treated with same protocol as cells plated on uncoated slides. To confirm the effectiveness of coating with a ECM constituent, and also to see its effect of adsorption of serum factors, such as FN and TSP, a representative coated chamber slides and noncoated slides were immunoperoxidase-stained without MC-plating after 24 hours incubation at 37 C with 400 μl of HBSS, complete medium or conditioned culture medium obtained from 75 sq cm flask of over-confluent MCs.

Immunoperoxidase Staining

Immunoperoxidase staining (avidin-biotin method) for cultured MCs on chamber slides was performed, on the day before re-feeding (ie, after 4 days serum starvation), and daily from 1 to 5 days after re-feeding with complete medium containing 20% FCS. After each chamber was washed three times with PBS containing 0.1 g/l $CaCl_2$, the upper plastic house of the chamber was removed so that the chambers of each slide were separated only by rubber seams. MCs outgrowths were fixed with absolute methyl alcohol at −20 C for 10 minutes, and washed three times with PBS. Each chamber was incubated with 50 μl solution of different primary antibodies raised in rabbits. Affinity-purified, monospecific antibodies to rat Col I, human Col III, and murine EHS sarcoma Col IV were produced by Drs. J. A. Madri and H. Furthmayr (Yale University School of Medicine), have been well-characterized and reported elsewhere,[19,20] and were provided by them. Affinity-purified, monospecific antibodies to human plasma FN and murine EHS sarcoma LM were also produced, characterized,[21] and provided from the same

sourse. These antibodies were demonstrated to cross-react with tissue antigens of fresh frozen rat kidney and cultured rat cells.[22,23] These antibodies were used at the dilution of 1:100 to 1:400 in PBS with 0.5% BSA (PBS/BSA), after prior titration. Rabbit monospecific antibody to human platelet TSP was produced and characterized[10,24] by Drs. G. J. Raugi and D. H. Lovett (University of California, San Francisco). Immunoperoxidase staining with this antibody of frozen sections of rat kidney showed focal, weak, granular staining in glomerular mesangium and occasionally in the wall of small vessels (data not shown). This pattern of staining in the rat kidney was similar to that with human kidney reported by Wight et al.[11] In the present study, it was used at a dilution of 1:100. After incubation with different primary rabbit antibodies for 1 hour at 22 C, slides were washed five times with PBS, and each chamber was incubated with 1:500 dilution of biotin-conjugated goat anti-rabbit IgG (heavy and light chain specific, Vector Laboratories, Burlingame, CA) for 30 minutes. After washing five times, each chamber was incubated in 1:200 dilution of horse radish peroxidase (HRP)-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA) in PBS for 15 minutes. After washing five times, 0.024% of 3-amino-9-ethylcarbazole (AEC) solution in 0.1 M sodium acetate buffer, pH 5.2, with 0.01% H_2O_2 was applied as the substrate of HRP for exactly 15 minutes at 22 C and was then washed out with warm tap water. After removing the rubber separations, the slides were counter-stained with Meyer's hematoxylin solution for 15 seconds, mounted with liquid glycerol gelatin (Sigma), and observed with a Leitz microscope.

The intensity of brown immunoperoxidase staining for ECM constituents, both in the cells and outside the cells, respectively, was scored semiquantitatively, using an arbitrary scale of four grades—(−), (+), (++), and (+++). The assessment of staining intensity was based on the inspection of more than ten visual fields for each stained specimen.

Immunoelectron Microscopy

For the immunoelectron microscopic study, chamber slides plated at 1000 cells per chamber were prepared. The staining for immunoelectron microscopy was performed on same time course as that for light microscopic immunoperoxidase staining. Each chamber was washed three times with chilled PBS with 0.1 g/l $CaCl_2$. MCs on chamber slides were incubated with freshly prepared PLP (periodate lysine paraformaldehyde) fixative[25] overnight at 4 C. Chamber slides were washed with chilled PBS, incubated in chilled 10% sucrose solution in PBS for 4 hours, 15% sucrose for 4 hours, and 20% sucrose overnight. The cells were permeabilized by incubation in 20% sucrose solution in PBS with 5% gelatin followed by dipping into liquid nitrogen for 1 minute, thawing, and washing three times with chilled PBS. To prevent nonspecific binding, chamber slides were incubated with 1:5 dilution of normal goat serum in PBS/BSA for 30 minutes. The same primary antibodies as mentioned above were applied to each chamber for 2 hours at 22 C. After washing five times with chilled PBS, horseradish-peroxidase (HRP)-labeled goat anti-rabbit IgG (heavy and light chain specific, Vector) was applied at a dilution of 1:400 for 1 hour at 22 C and washed five times. MCs were postfixed in 2% glutaraldehyde in PBS at 4 C for 30 minutes, and washed five times. They were incubated in 0.05% 1,3-diaminobenzidine (DAB) in 0.05 M TRIS-HCl buffer, pH 7.6, for 10 minutes at 22 C to facilitate the access of DAB to HRP, and then incubated in another DAB solution with 0.01% H_2O_2 for 10 minutes. The slides were washed five times with PBS and postfixed with 1% osmium tetraoxide in PBS for 45 minutes. After removing the rubber seams, the slides were dehydrated with graded ethanol. For embedding MCs in epoxy resin, the "pop-off" method[26] was employed to the slides, ie, sites of immunoreaction on the slides were covered by plastic capsules 0.6 cm in diameter filled with epoxy resin. After incubation overnight at 60 C for 12 hours, partially hardened epoxy resin were removed, or "popped off," from glass slides. The hardened block of epoxy-resin was cut into small pieces, and re-embedded to orient the MC layers vertical to cutsurface. Semithin sections were cut with a glass knife, and ultrathin section were cut with a diamond knife using a LKB ultramicrotome. The unstained specimens were transfered to copper grids and examined in an electron microscope (Philips, Eindhoven, The Netherlands).

Table 1. *Time Interval Between Plating and MC Confluency in Chamber Slides as a Function of Initial MC Plating Density*

Plating density (MCs/chamber)	Time interval of MC confluency after re-feeding (days)
4000	0.5
2000	1
1000	2.5
500	3.5
250	4

Results

Growth of MCs and Time of Confluency

MCs attached and grew well independent of the employed initial plating density. As shown in Table 1, MCs that were plated at a higher cell density became confluent

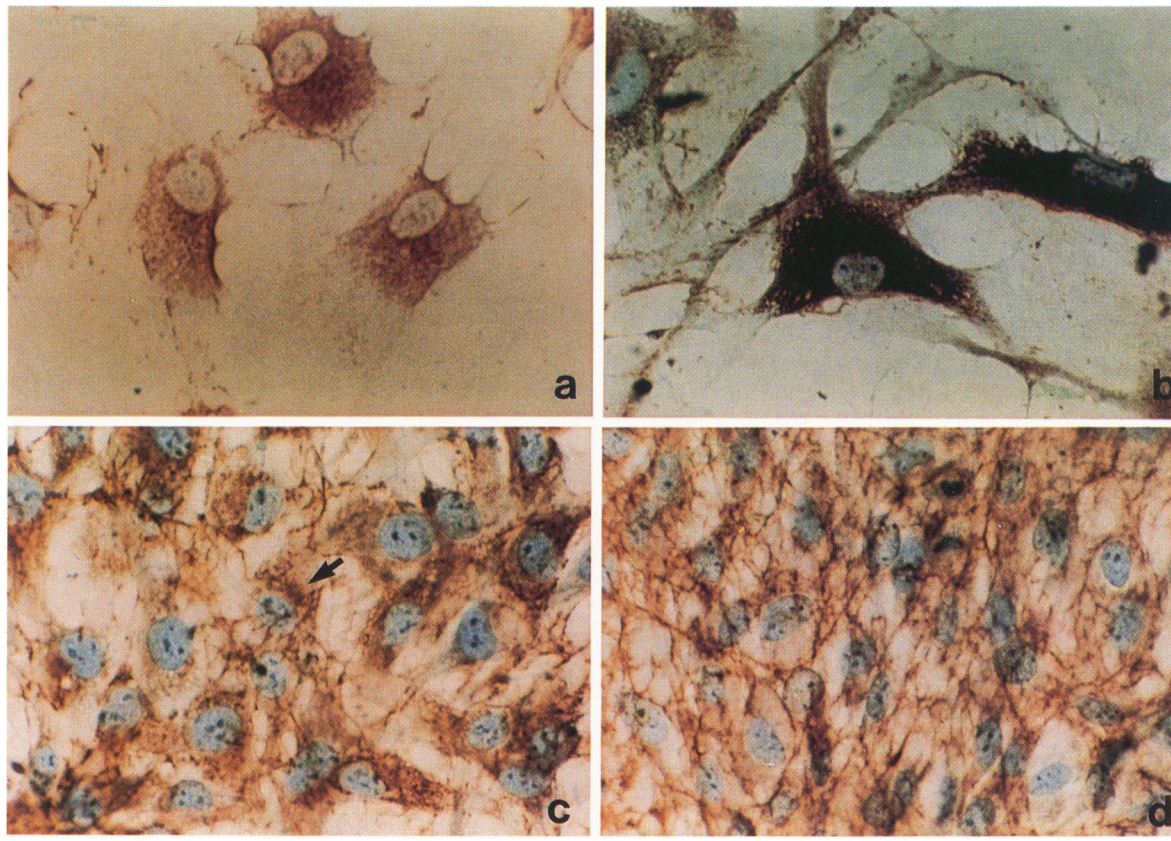

Figure 1. *Immunoperoxidase staining for FN on the slide initially plated at the density of 1000 cells per chamber.* **a:** *Before re-feeding with complete medium. Diffuse intracellular staining of ER pattern is seen (×330).* **b:** *One day after re-feeding. Staining intensity increases markedly (×330).* **c:** *Three days after re-feeding when MCs reached confluency. Extracellular deposition appears, while intracellular staining decreases. Focal paranuclear staining of Golgi pattern is noted (arrow) (×330).* **d:** *Five days after re-feeding when MCs grew to confluency. The network of extracellular FN aggregations is more abundant, while intracellular staining is diminished (×330).*

earlier than those in chamber slides plated at a lower concentration. Thus, MCs plated at 4000 cells per chamber became confluent within half a day, whereas confluency was not reached until 4 days in chambers receiving only 250 MCs.

Immunoperoxidase Staining

The localization of ECM antigens stained by the immunoperoxidase technique on MC culture slides demonstrated two clearly different staining patterns. One was intracellular staining, confined to the MC cytoplasm without nuclear staining. The other was staining of fine or coarse aggregations of ECM antigens around MCs, not related to cellular configuration. The latter was easily assessed to be extracellular staining by light microscopy, and this localization was further confirmed by immunoelectron microscopy (see below). Both intracellular and extracellular staining were observed for FN, LM, Col III, and Col IV, whereas staining for Col I and TSP was limited to intracellular localization. The time course of localization of ECM antigens varied and three different patterns were observed. First, for FN and Col III, intracellular and extracellular localizations both were seen during the time course, with their reciprocal change that occurred around the time of confluency. Second, for Col IV and LM, the reciprocal change in intracellular and extracellular localization occurred a little earlier than that of FN and Col III. Third, the staining pattern of Col I and TSP was characterized by strictly intracellular staining without extracellular aggregation at any time point.

Staining for FN and Col III

The stainings of MC outgrowths for FN on slides initially plated with 1000 cells per chamber are shown in Figure 1. On the day before re-feeding of MCs with complete medium, intracellular staining for FN and Col III was observed. Intracellular staining was diffuse (staining of the endoplasmic reticulum or ER pattern) and finely granular without any focal condensation (Figure 1a). One day after

re-feeding, as MCs increased in number, intracellular staining of the ER pattern greatly increased in intensity (Figure 1b). Two or three days after re-feeding, as MCs reached confluency, intracellular staining gradually decreased in intensity. Some MCs showed focal, paranuclear staining in a Golgi staining pattern. In parallel to the decrease of intracellular staining, fine, threadlike extracellular aggregations appeared and gradually increased in intensity (Figure 1c). Intracellular staining of focally crowded MCs was weaker than that of cells at relatively sparse density, and extracellular staining of the former was stronger and coarser than that of the latter. Four and five days after re-feeding, when MCs had reached confluency, intracellular staining decreased to become weak or negative, while extracellular staining markedly increased in staining intensity and abundance (Figure 1d). Fine aggregations of extracellular staining became coarser, forming an irregular network surrounding densely packed cells.

On the slides initially plated at lower density (500 and 250 cells per chamber), a similar pattern of immunoperoxidase staining was observed as found on slides plated at 1000 cells. Because MCs at low plating density reached confluency later, however, the decrease of intracellular staining and appearance of extracellular staining also occurred at a later time point. On the slides initially plated at 2000 and 4000 cells per chamber, intracellular staining was weaker on the day before and after re-feeding than in slides plated at lower density, and it became almost negative 2 or 3 days after re-feeding. In these high-density preparations, foci of fine extracellular staining was seen, even before re-feeding. These became coarser and increased in staining intensity and amount as the days passed after re-feeding.

Semiquantitative assessment of the changes in staining intensity for FN on slides initially plated at 250, 1000, and 2000 MCs per chamber is shown in Figure 2. It is evident that, after re-feeding of sparsely populated MC cultures, intracellular staining first increases and then starts to decrease around the time of MC confluency when the first extracellular staining become appreciable. Extracellular staining then continually increases in amount and intensity. This general time course is independent of the initial MC plating density, ie, this reciprocal change in intracellular and extracellular localization occurs around MC confluency.

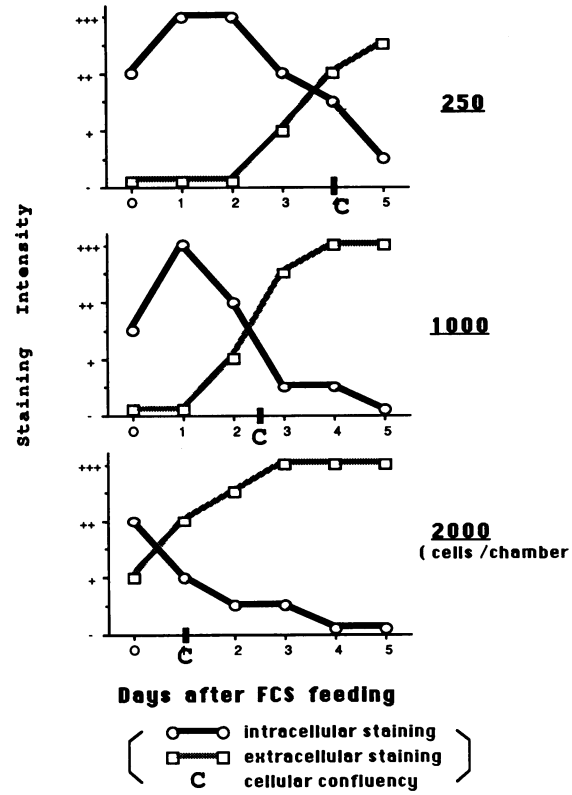

Figure 2. *Time course of semiquantitative assessment of intensity of intracellular vs. extracellular staining for FN on chamber slides, which had initial plating densities of 250, 1000, and 2000 cells per chamber. These data are from a representative single experimental series. Extracellular staining appears shortly before or at MC confluency, when intracellular staining decreases. Similar time course of staining was seen for Col III.*

Staining for Col IV and LM

Immunoperoxidase staining of MC outgrowths for Col IV on slides initially plated at 1000 cells per chamber is shown in Figure 3. On the day before re-feeding, diffuse intracellular staining in an ER pattern was observed for Col IV and LM. One day after re-feeding, intracellular staining increased markedly in intensity. Of note is that, in addition to diffuse staining of ER pattern, a focal paranuclear staining (Golgi pattern) for Col IV and LM was observed in many cells (Figure 3a). This was observed even before MC confluency at a time when few MCs exhibited a Golgi staining pattern for FN and Col III. Two days after re-feeding, when MC outgrowths were still subconfluent, intracellular staining decreased in intensity in many cells, while fine aggregations of extracellular staining for Col IV and LM began to appear (Figure 3b). The appearance of extracellular aggregations of Col IV and LM was found about 1 day earlier than that of FN and Col III. Three to five days after re-feeding, intracellular staining decreased to become undetectable, and extracellular aggregations increased in amount and became coarser to form a dense irregular network around the cells (Figure 3c).

On the slides initially plated at lower density (500 or 250 cells per chamber), similar patterns in intracellular and extracellular staining for Col IV and LM were observed. The decrease of intracellular staining and the appearance of extracellular staining occurred and increased later, as MCs on the slides at 500 and 250 cells reached conflu-

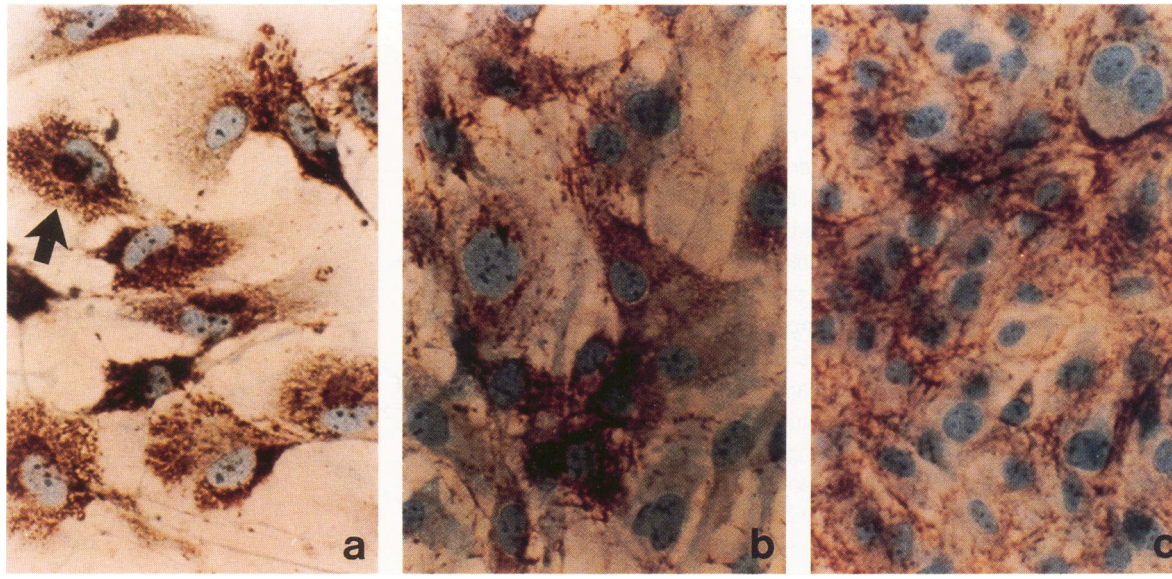

Figure 3. *Immunoperoxidase staining for Col IV on the slide initially plated at the density of 1000 cells per chamber.* **a:** *One day after re-feeding with complete medium. In addition to diffuse intracellular staining of ER pattern, focal paranuclear staining of Golgi pattern (arrow) is seen before confluency (×330).* **b:** *Two days after re-feeding when MCs were nearly confluent. Fine aggregations of extracellular staining are seen around subconfluent MCs, while intracellular staining is reduced markedly (×330).* **c:** *Four days after re-feeding when MCs were grown after confluency. Thick, extracellular aggregation of Col IV is marked (×290).*

ency later than those at 1000 cells. On the slides initially plated more densely (2000 or 4000 cells per chamber), fine extracellular staining was found even before re-feeding. After re-feeding it increased greatly in amount, forming coarse aggregations, while intracellular staining decreased rapidly.

Semiquantitative assessment of changes in staining intensity for Col IV on the slides initially plated at 1000 cells per chamber is shown in Figure 4, with that for Col III and Col I for comparison. In this figure, extracellular staining for Col IV increases and intracellular staining decreases earlier than that for Col III.

Staining for Col I and TSP

In contrast to the four ECM antigens mentioned above, immunoperoxidase staining for Col I and TSP never showed any significant extracellular aggregation at any time point or cell density. On the day before re-feeding, staining of these two antigens was localized diffusely in the MC cytoplasm (ER pattern). Staining for Col I was finely granular, whereas that for TSP was more homogeneous. After re-feeding, cytoplasmic staining intensity of both antigens was clearly increased (Figure 5a). Around the time of confluency, in addition to diffuse staining of the ER pattern, focal paranuclear aggregations of staining (Golgi pattern) were observed frequently. After confluency, intracellular staining for both antigens remained strong, while extracellular aggregation was not appreciated (Figure 5b). Intracellular staining for Col I and TSP in the Golgi pattern remained prominent in addition to abundant ER staining. Figure 4 illustrates the semi-quantitative assessment of these changes of staining for Col I on the slides initially plated at 1000 cells per chamber.

Effect of Ascorbic Acid on ECM Production and Distribution

Because ascorbic acid has been demonstrated to influence collagen secretion,[27] the effect of the addition of 50 μg/ml ascorbic acid on MC secretion of matrix was examined. Because a preliminary study demonstrated that MCs could grow with 50 μg/ml ascorbic acid at higher cell densities (1 or 2 days after confluency) but not at lower cell densities (before confluency), complete medium containing ascorbic acid was added only onto the chambers with over-confluent MCs. Two days after adding ascorbic acid, slides were stained for each ECM antigens. The staining pattern (localization of each ECM antigens) and intensity showed no difference between ascorbic acid treated cells and nontreated cells. Extracellular staining for FN, Col III, LM, and Col IV was observed, while staining for Col I and TSP remained intracellular with no extracellular aggregation.

Effects of Precoating Substrata with ECM

Representative chamber slides without MCs were shown to be effectively and specifically coated with each ECM

antigen by immunoperoxidase staining with the five antibodies (FN, LM, Col I, Col III, or Col IV). No crossreactivity was observed between collagen antigens and antibodies. After incubation with complete medium or conditioned culture medium from a 75 sq cm flask with over-confluent MCs, diffuse weak homogeneous staining for FN was found on the slides coated with LM or Col IV. Barely detectable staining for TSP was seen on FN-, LM-, or Col IV-coated slides. The staining pattern of nonspecifically bound constituents was diffuse and hazy and did not resemble the fine and coarse extracellular aggregates of matrix constituents seen with postconfluent cultures of MCs or in the following result. Nonspecific binding of other ECM antigens was not detected.

MCs plated at 1000 cells per chamber seemed to attach well on slides precoated with different ECM antigens when assessed by phase contrast microscopy.[28] However, MCs initially plated at 1000 cells per chamber became confluent 2 to 3 days after re-feeding with complete medium after 4 days starvation.

On chamber slides coated with FN, Col I, Col III, or denatured BSA, the staining pattern, time course, and distribution of each ECM antigen did not show appreciable differences from results obtained on noncoated chamber slides (Figure 6a). In contrast, on slides coated with Col IV or LM, significant differences in the staining pattern were observed. On the day before re-feeding, when subconfluent MCs had been serum-starved for 4 days, fine extracellular staining for FN, LM, and Col III was observed on Col IV-coated slides. Similarly, extracellular FN, Col III and IV were observed on LM-coated slides. This was in addition to the presence of intracellular staining for these ECM antigens. At one day after re-feeding, both intracellular and extracellular staining was markedly increased in intensity, even though MCs were far from confluency (Figure 6b, c). Fine aggregations of extracellular staining were turning into coarser aggregations. Extracellular localization before MC confluency was more prominent for FN, Col III, and Col IV on LM-coated slides than for FN, LM, and Col III on Col IV-coated slides. Focal paranuclear aggregation of intracytoplasmic ECM antigens (Golgi staining pattern) also was observed before MC confluency (Figure 6c). On the days at and after MC confluency, intracellular staining decreased, and extracellular staining increased further and formed coarser aggregations. These staining patterns were similar to those at late time points on noncoated slides. With respect to the Col I and TSP localization, extracellular aggregation of these antigens was not observed even on the slides coated with Col IV or LM. Semiquantitative assessment of changes in staining intensity also was performed. Figure 7 shows the results of the changes in staining intensity for FN on Col IV-coated slides and on BSA-coated slides as a control.

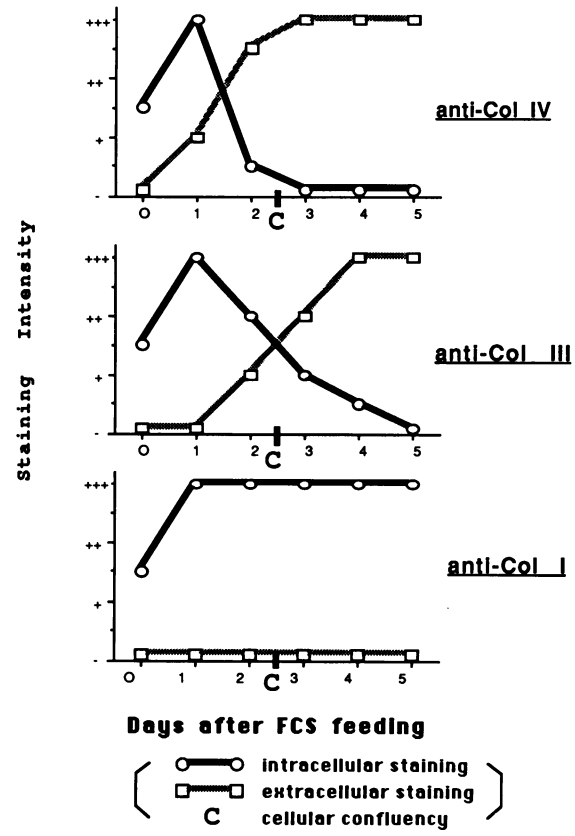

Figure 4. *Time course of semi-quantitative assessment of intensity of intracellular* vs. *extracellular staining for Col IV, Col III, and Col I on slides initially plated at a density of 1000 cells per chamber. Extracellular staining for Col IV appeared earlier than that for Col III, and diminution of its intracellular staining earlier. In contrast to Col IV and Col III, no appearance of extracellular staining nor diminution of intracellular staining for Col I is seen. Time course of the staining for LM was similar to that for Col IV, and that for TSP was similar to that for Col I.*

Immunoelectron Microscopy

Although no reaction product was seen in control specimens, localization of ECM antigens was evident in specimens stained with antibodies for FN, LM, Col I, Col III, Col IV, and TSP. At a time of low MC density, almost all MCs had reaction product in the cytoplasm. All ECM antigens were localized to the inner membrane or inner space of the endoplasmic reticulum (ER, Figure 8a). Fine, focal staining along the cell membrane also was seen for FN, LM, Col IV, and TSP.

At MC confluency, some cells had staining of the ER for FN, LM, Col III, and Col IV, but other cells did not. More MCs showed membrane staining for these four ECM antigens and membrane staining increased in extent and thickness. Small, irregular aggregates were formed and unevenly distributed along the outer aspect of the cell membrane (Figure 8b). With Col I and TSP, almost all cells had reaction product in the ER. Membrane-bound stain-

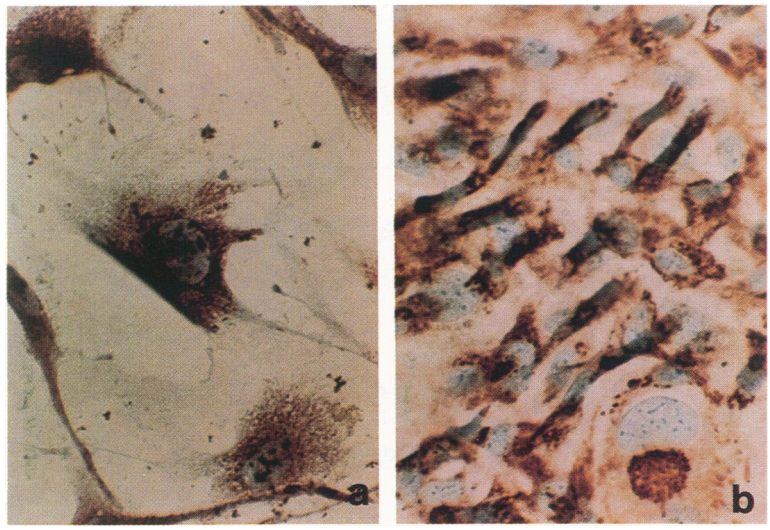

Figure 5. *Immunoperoxidase staining for Col I on a slide initially plated at a density of 1000 cells per chamber.* **a**: *One day after re-feeding (×330).* **b**: *Five days after re-feeding when MCs have reached confluency (×330). Staining is strictly intracellular, both before and after MC confluency.*

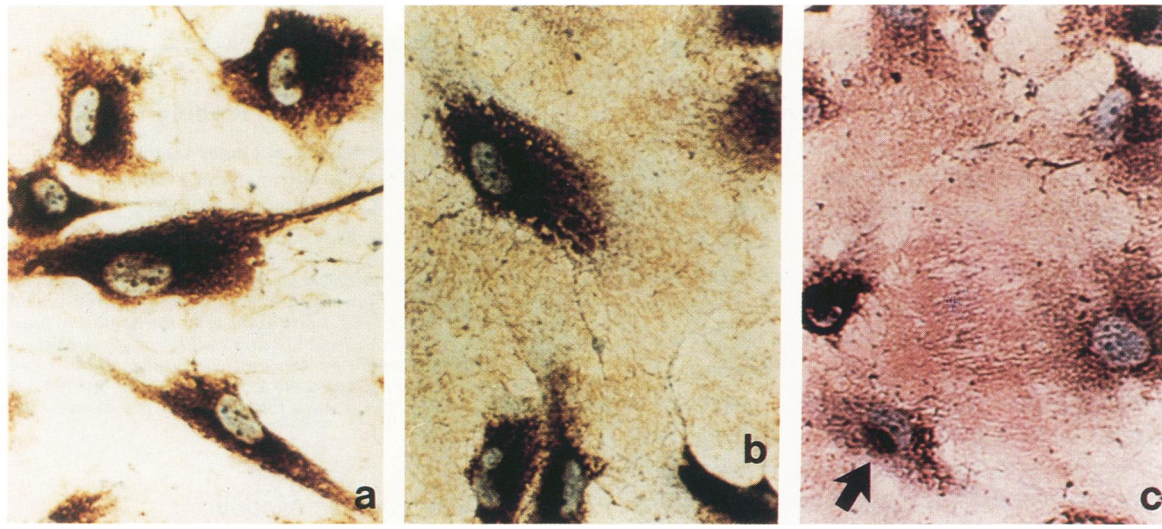

Figure 6. *Staining for FN in subconfluent MC outgrowths on an uncoated slide (***a**, *×330) and Col IV-coated slide (***b**, *×330), and staining for Col III on LM-coated slide (***c**, *×330). Extracellular aggregations of FN on Col IV-coated slides and of Col III on LM coated slides are seen in MC outgrowths before confluency, but not on uncoated slide. Focal paranuclear staining of Golgi pattern (arrow) is seen.*

ing for Col I and TSP also was found occasionally but no extracellular aggregates were seen.

After confluency, MCs were frequently two or three-cell layered, and large amounts of extracellular reaction product for FN, LM, Col III, and Col IV accumulated between MCs, forming large, inhomogeneous intercellular aggregates (Figure 8c). Few multilayered cells had reaction product in the ER. In contrast to the other ECM antigens, cytoplasmic Col I and TSP continued to be present in the ER, and foci of thin, membrane-bound reaction products were found occasionally.

Discussion

Expansion of the extracellular matrix (ECM) is seen frequently in glomerular disease. It is especially prominent in chronic progressive glomerular lesions,[1,29,30] such as chronic glomerulonephritis and diabetic nephropathy. Increasing ECM expansion is associated with a reduction of the glomerular filtration surface area and, thus, a decrease in renal function. Glomerular ECM is formed by intrinsic glomerular cells, ie, epithelial cells, endothelial cells, and MCs. While epithelial cells and endothelial cells produce matrix constituents of the basement membrane type (eg, Col IV, Col V, and LM),[31,32] MCs have been shown to be capable of also producing ECM constituents of the interstitial type (eg, Col I, Col III, and FN).[1,3,14,31,33] These ECM components have been described to be present in sclerosing glomeruli studied by immunohistochemistry on renal tissue sections.[30,34,35] They are thought to be formed by MCs or infiltrating fibroblasts.[35] The

mechanisms that regulate the formation of ECM constituents by MCs must therefore be elucidated for a better understanding of the process of ECM accumulation in diseased glomeruli.

In the present study, we examined, *in vitro,* the production and secretion of ECM constituents by cultured rat MCs. Using immunoperoxidase cytochemistry and immunoelectron microscopy, MCs were demonstrated to produce various ECM constituents of both the basement membrane type and the interstitial type. Our findings confirm and extend the results of others using different techniques of ECM analysis or different culture condition.[3,14] In addition, we examined the presence and distribution of ECM constituents as affected by time, MC growth activity, and cell density.

After 4 days of serum starvation, growth-arrested MCs showed weak intracellular staining for all ECM antigens examined. The staining increased greatly when MCs started to proliferate actively in the presence of full medium containing 20% FCS. Thus, the phenotypic expression of ECM production paralleled the proliferative activity of MCs, indicating that active protein synthesis and DNA replication were stimulated simultaneously. This pattern was observed for all tested ECM constituents. It appears, therefore, that the poorly defined mixture of factors contained in 20% FCS stimulates both proliferation and differentiated function of cultured MCs. It is unclear, however, whether a single MC growth factor, such as PDGF,[15] IL-1,[13] or arginine vasopressin,[16] affects both MC proliferation and matrix production, or whether these two aspects of cell behavior are modulated separately by different stimuli. The finding that intracellular staining of MCs for FN and Col III as well as Col IV and LM diminished around confluency when MCs are still actively proliferating suggests that the two cell functions are regulated independently, though this interpretation must take into account the role of secreted ECM proteins as solid phase autocrine factors.

With regard to intracellular *vs.* extracellular localization of ECM proteins, pronounced changes in distribution of antigens were observed for FN, Col III, Col IV, and LM when studied before, during, and after MC confluency. At low cell density staining for these antigens was strictly intracellular. This was independent of the time after refeeding with complete medium in chambers that were initially plated at different densities. Extracellular localization of Col IV and LM was seen first, followed by FN and Col III, which appeared extracellularly around the time of MC confluency. Just before and during appearance of extracellular localization, focal paranuclear staining in a Golgi pattern was observed frequently. This morphology appears to reflect the transport and processing of ECM constituents in the Golgi apparatus before extracellular deposition. These observations indicate that, as cellular confluency is reached and cell–cell contact is established,

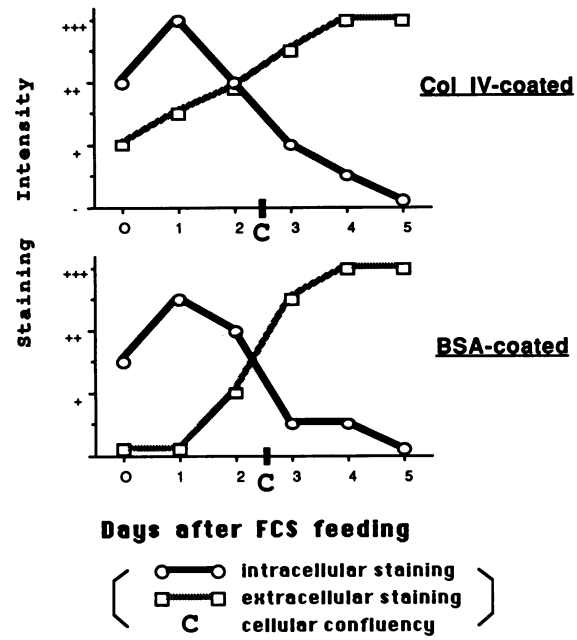

Figure 7. *Time course of semiquantitative assessment of intensity of intracellular* vs. *extracellular staining for FN on Col IV-coated slides or BSA-coated slides. Earlier appearance of extracellular staining is seen on Col IV-coated slides. Similar time course as the former was noted in the staining for LM, Col III on Col IV-coated slide, and for FN, Col III and Col IV on LM-coated slide, but not on slides coated with FN, Col I, Col III, or BSA.*

MCs are stimulated to process and secrete these four ECM constituents, which are eventually deposited between and under the cells. All of these events are independent of the time after the initiation of MC proliferation by re-feeding complete culture medium. This interpretation also is supported by the observation that, even when MCs are subconfluent, fine extracellular aggregations of ECM staining were found only in the areas of crowded MCs, which reflected focal confluency.

After confluency, extracellular aggregates of Col IV, LM, FN, and Col III increased in extent, while intracellular antigens decreased. The electron microscopic observations confirmed these results further. In nonconfluent, single-layered MCs, ER staining was prominent, and only focal, fine membrane-associated staining was noted. Once MCs had formed cell–cell contact, membrane-bound ECM staining increased in amount and extracellular aggregates appeared. When MCs had grown to a multilayer, thick aggregations of ECM antigens between the cells markedly increased, while ER staining diminished considerably. These events also were independent of the date after re-feeding on differently populated chamber slides. The extracellular aggregation of ECM antigens rapidly increased during and after MC confluency, consistent with extracellular secretion and organization of matrix components. While some of the extracellular localization might

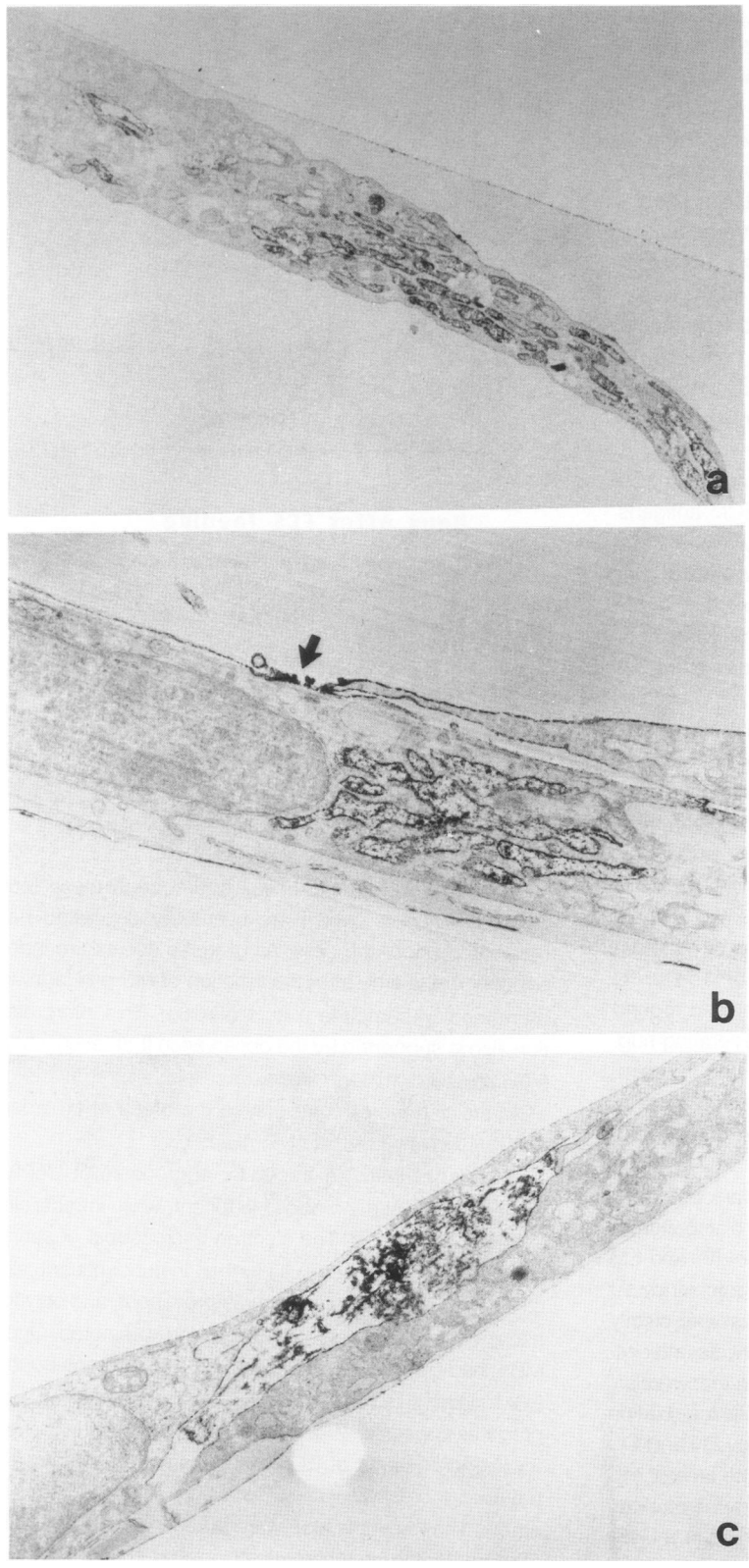

Figure 8. *Immunoelectron microscopy for FN.* **a:** *One day after re-feeding (×7200).* **b:** *Three days after re-feeding (×12,000).* **c:** *Four days after re-feeding (×10,800). Before MC confluency (a), heavy ER staining are seen. At MC confluency (b), in addition to ER staining, irregular membrane-bound extracellular reaction products are seen (arrow). After MC confluency (c), large extracellular aggregations of FN are observed between MCs, while ER staining decreases markedly.*

be attributed to nonspecific binding of soluble antigens derived from the fetal calf serum (especially with FN and TSP), this seems unlikely because slides incubated 24 hours with complete medium or MC conditioned culture medium (which contains soluble ECM components) without MC plating onto them failed to demonstrate any ag-

gregated ECM antigens. Furthermore, the aggregations were formed and entrapped extracellulary between the cells as well as under them. These findings are consistent with the concept that the secreted extracellular ECM components interact with specific MC membrane receptors to target delivery and to regulate their own synthesis, secretion, and extracellular organization with mechanisms that include an autocrine feedback system as a solid phase. To fully test this hypothesis, however, quantitative measurements of the ECM proteins are required not only in the MC outgrowth but also in culture supernate.

In contrast to the other four matrix components examined, no extracellular aggregation of Col I and TSP was observed even several days after MC confluency. The paranuclear Golgi pattern staining for Col I and TSP was found continuously during and after cellular confluency. By electron microscopy, staining for Col I and TSP was noted in the ER and only focally associated with the cell membrane. Other investigaters have shown that Col I[3] and TSP[10] are secreted by MC into culture medium, and while TSP was found to be membrane-bound,[10] these studies have not shown whether these ECM antigens are incorporated into the underlying organized matrix. Considering these results, our finding of persistent ER, Golgi, and cell membrane-associated staining suggests that MCs in two-dimensional culture conditions continuously produce and secrete Col I and TSP into the supernate without being organized into the extracellular matrix. The absence of extracellular aggregations of these proteins close to MCs might result in an absence of down regulation of production and secretion of Col I and TSP by MCs even after confluency and explain why some studies have shown that Col I is the predominant ECM protein produced by MC in culture.[3] Clearly, further studies are required to assay MC conditioned media for the appearance of soluble ECM constituents that may have been secreted by MCs into the supernate.

In this study, the reason for the lack of extracellular aggregation of Col I and TSP is unclear. Addition of ascorbic acid to prevent incomplete hydroxylation and intracellular recycling of collagen proteins secondary to vitamin C deficiency did not result in extracellular aggregation. Haralson and coworkers[3] analyzed the amount and distribution of collagen types, using ^{3}H-proline incorporation, and reported that, while Col I comprises 95% of total collagen protein synthesized by MCs, 80% was in the form of α1(I). This led these investigators to suggest that Col I α1 trimer [α1(I)$_3$] is preferentially secreted rather than the usual heterotrimer form of Col I [α1(I)$_2\alpha$2(I)]. This unusual pattern of secretion might also be responsible for the lack of incorporation of Col I into an organized matrix with continued secretion in the medium.

The results of present study suggest that, at MC confluency, cell–cell contact seemed to trigger the secretion of FN, Col III, Col IV, and LM, and organization of an extracellular matrix. Cell–cell contact plays an important role in the modulation of the behavior of many cells. For example, cell–cell contact of cultured MDCK cells has been found to trigger redistribution of Na,K-dependent ATPase, ankyrin, and fodrin with development of cell polarity.[36–38] Although the exact mechanism activated by cell–cell contact is not yet fully described, there is strong evidence that interaction of integral membrane proteins and the cytoskeleton is involved. Cell–cell contact of MCs could induce stabilization of membrane proteins, such as the receptors for Col IV or LM, by the cytoskeleton in a manner analogous to Na,K-dependent ATPase in MDCK cells.[36] This would allow specific binding of ECM constituents by such receptors, which in turn could influence ECM protein synthesis and secretion through a signal transduction mechanism.

It is of interest that Col IV and LM were the ECM components first detected as extracellular deposits on uncoated slides and that extracellular secretion of FN and Col III occurred subsequently. This observation suggested that extracellular Col IV or LM or both might act as a trigger for the secretion of the other components. This concept was confirmed with the experiments conducted on slides coated with matrix components. Coating of culture chamber with Col IV or LM greatly facilitated the extracellular secretion and deposition of other ECM constituents at the time when MCs had not reached confluency. Experiments designed to test whether nonspecific binding occurred showed weak and homogeneous, but not aggregated, staining for FN and TSP only on some slides. This pattern was different from aggregated staining observed for ECM staining on Col IV- or LM-coated slides with MCs outgrowth. Similar extracellular aggregation on LM- or Col IV-coated slides also has been observed by immunofluorescent staining of cultures of subconfluent endothelial cells by Madri and coworkers.[23] In our study, no stimulatory effects were evident on slides coated with Col I, Col III, and FN. The difference of the effect of substrata could not be attributed to differences in plating efficiency, since previous studies[28] have shown that Col I, III, and IV provided similar attachment properties (60%) for MCs, while LM was somewhat less efficient (25%). Since Col IV and LM are basement membrane components, whereas FN, Col I, and Col III are interstitial proteins, it is probable that basement membrane organization plays a primary role in the regulation of MC secretion of other ECM glycoproteins.

It has been shown that the ECM influences the behavior of cells, including proliferation,[13,28,30,39–41] differentiation,[18,40] and secretion.[23,42] Regulation of cell behavior by ECM has been observed in several cell types, such as endothelial cells,[23] epithelial cells,[42] hematopoietic cells,[40] smooth muscle cells,[44] as well as MCs.[28,30,41] Davis and coworkers[43] have reported that the nature of the underlying substratum affects the synthesis of collagen types by

Ito cells. As mentioned above, Madri and coworkers[23] demonstrated that LM or Col IV substrata facilitate marked, extracellular secretion of other ECM antigens. Recently, Grond and coworkers[41] observed that Col IV, LM, FN, and TSP are the ECM constituents found in areas of proliferating MCs in prolonged culture. The present findings, taken in the context of the results of other investigators, emphasize the important role that the composition and organization of the ECM may play in the modulation of MC behavior in the glomerulus *in vivo*. It is presently unclear, however, whether and how these observations in cell culture may pertain to cell-matrix interactions *in vivo*. One may hypothesize that in the glomerulus, *in vivo,* an injury may cause MC proliferation and that this, perhaps augmented by greater MC density and facilitated cell–cell contact, may affect synthesis, composition, and secretion of ECM proteins. Conversely, changes in the composition of the mesangial ECM, as reported in several disease states,[7,30,35] could affect both MC proliferation and production of ECM components. The mechanisms operating in such cell-matrix interactions and their potential role in tissue remodeling in diseased glomerulus and in the pathogenesis of progressive glomerular sclerosis, remain to be delineated further.

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