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Increased expression of extracellular matrix proteins and decreased expression of matrix proteases after serial passage of glomerular mesangial cells

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SUMMARY

The cellular events causing pathological extracellular matrix (ECM) accumulation in vivo are not well understood. Prolonged serial passage of several cell types in culture leads to increased production of extracellular matrix (ECM) proteins, but the mechanism for these putative fibrotic changes is not known. Here, human fetal glomerular mesangial cells were subjected to serial passage (P) in culture and the expression of ECM proteins, proteases and protease inhibitors was comprehensively evaluated. From P11 through P14, a series of phenotypic changes occurred. Steady-state expression of mRNA for α1 chains of type III and type IV (but not type I) collagen, and for laminin β1 and γ1, increased 2- to 8-fold, while expression of mRNA for interstitial collagenase (MMP-1) and gelatinase A (MMP-2) virtually ceased. Expression of tissue-type plasminogen activator (tPA) mRNA also decreased markedly. Expression of mRNA for the tissue inhibitor of metalloproteinases (TIMP)-1, and of the smaller of two mRNA species for the PA inhibitor PAI-1, ceased by P14. There was a switch in expression of the two species of TIMP-2 mRNA: whereas the ratio of signal intensity comparing the 3.5 kb mRNA species to the 1.0 kb species was 5:1 up to P11, it was reversed (1:5) at P14 and later. Serial passage also led to changes in protein expression, with increased type IV collagen and laminin, but decreased interstitial collagenase and gelatinase A. The cells showed a progressive increase in staining for type IV collagen. These findings define the appearance of a matrix-accumulating phenotype in later-passage mesangial cells.

Matrix expansion in vivo has been associated with increased transforming growth factor (TGF)-β synthesis; the cells were found to show at least 5-fold increased expression of TGF-β1 mRNA from P8 to P16. However, treatment of P9 or P10 cells with graded doses of TGF-β1 increased expression of both collagen IV and gelatinase A mRNA and did not alter the ratio of signal intensity for TIMP-2 mRNA species. Thus, assumption of a matrix-accumulating phenotype by these cultured fetal glomerular mesangial cells is not accelerated by exogenous TGF-β. These data describe an in vitro model of mesangial cell matrix turnover in which matrix accumulation could result from a concerted increase in ECM synthesis and decrease in ECM degradation.

Key words: Glomerulosclerosis, Mesangial cell, Collagen, Extracellular matrix, Extracellular matrix protease

INTRODUCTION

Under certain pathological conditions, the accumulation of excess extracellular matrix (ECM) in tissues leads to dysfunction. This matrix accumulation, part of the process of sclerosis, may cause thickening of basement membranes, disruption of normal cell-cell interactions, and loss of tissue compliance or elasticity in such conditions as atherosclerosis of the blood vessels (Raines and Ross, 1991), pulmonary fibrosis (McGowan, 1992), liver cirrhosis (Rojkind, 1991), and destruction of the glomerulus that filters blood in the kidney. Glomerulosclerosis involves glomerular structural collapse and ECM accumulation (Thoenes and Rumpelt, 1977). It may be a primary manifestation of glomerular disease, but it also represents a common series of events by which function is lost in a variety of kidney diseases (Schnaper, 1996). Although several factors have been implicated in causing glomerulosclerosis, the underlying cellular mechanisms which lead to matrix accumulation are less well understood. Most studies of glomerular scarring have emphasized the role of increased ECM synthesis in matrix expansion (Adler et al., 1986; Brazy et al., 1991; Kopp et al., 1992). However, it is likely that the net rate of matrix turnover is more important than the absolute rate of ECM protein synthesis alone. Thus, increasing attention

has been focused on the rate at which extracellular matrix degradation occurs, since the balance between synthesis and degradation of ECM components determines the accumulation or loss of matrix (Couchman et al., 1994).

The two most abundant structural proteins in the glomerular ECM are type IV collagen and laminin. These proteins are the major matrix components that accumulate in progressive glomerular disease. In addition, type I and type III collagen may accumulate in the interstitium (Jones et al., 1991) or the glomerulus (Striker et al., 1984). Two major proteolytic pathways are important in degradation of these molecules. In one, the matrix metalloproteinases (MMPs), a family of neutral zinc proteases, degrade the different ECM components with varying specificities (Birkedal-Hansen, 1993). The most well-studied of these are the collagenases and gelatinases that degrade interstitial/fibrillar (types I and III) and basement membrane (type IV) collagens, respectively. MMP activity is blocked by a group of endogenous inhibitory proteins termed tissue inhibitors of metalloproteinases (TIMPs) (Liotta et al., 1991). The second proteolytic pathway important in ECM degradation includes both the plasminogen activators that convert plasminogen to plasmin, and plasmin itself. These serine proteases are important in laminin degradation (Liotta et al., 1981). Plasminogen activators are blocked by the plasminogen activator inhibitors (PAIs) (Collen, 1980). Together, these two proteolytic systems serve as the major pathways of degradation affecting matrix turnover. Both have been implicated in matrix degradation by glomerular cells (Baricos et al., 1995). Changes in the amount of ECM proteases produced in the tissue, in their activity, or in the ratios between proteases and their inhibitors, should have a significant effect on the net accretion of ECM proteins in the kidney. Support for this hypothesis is found in the observation that, in rats induced to develop glomerulosclerosis by administration of puromycin aminonucleoside, progressive interstitial fibrosis (Jones et al., 1991) and glomerular sclerosis (Nakamura et al., 1994) are accompanied by shifts in the balance between ECM protein synthesis and degradation that would favor matrix accumulation; amelioration of the disease progression is associated with a reversal of these changes (Nakamura et al., 1994).

The cellular mechanisms by which the balance in matrix turnover is altered in glomerulosclerosis is not known. We propose that specific alterations in cell structure and function are associated with matrix accumulation. Thus, by determining characteristics of matrix-accumulating cells, it may be possible to identify cellular events that are potentially related to the pathogenesis of glomerulosclerosis. Mesangial cells occupy a central position in the renal glomerulus at the location where matrix accumulation is first noted in classical focal glomerulosclerosis (Schnaper, 1996). In the studies described here, human fetal mesangial cells were subjected to a prolonged series of passages in culture. This manipulation led to assumption of a matrix-accumulating phenotype, associated with increased expression of ECM components but decreased expression of several ECM proteases at the level of both mRNA and protein. In addition, expression of different mRNA species for two protease inhibitors varied with serial passage. These changes are not caused by exogenous transforming growth factor (TGF)-β1, a known stimulus of the sclerotic process. Instead, this phenotypic change by the cultured cells appears to result from another effect, yet to be determined. This model should prove useful for investigating the cellular mechanisms by which changes that contribute to matrix accumulation occur in vivo.

MATERIALS AND METHODS

Unless otherwise stated, all materials used are reagent grade and were purchased from commercial sources. Active, human recombinant TGF-β1 produced in CHO cells was purchased from R&D systems, Minneapolis, MN.

Culture and passage of the glomerular mesangial cells

Mesangial cells were isolated by differential sieving of minced glomerular tissue (Harper et al., 1984) obtained from a fetus after an elective therapeutic abortion at 14-15 weeks' gestation. The cells in this isolate, termed FMC2, were confirmed to be mesangial by morphological criteria, by the presence of abundant actin microfilaments, and by the absence of staining for cytokeratin and factor VIII-related antigen (Weeks et al., 1991). Confluent cultures of cells were split 1:5 in DMEM/Ham's F12 medium supplemented with 20% heat-inactivated fetal calf serum (Hyclone; Logan, UT), glutamine, penicillin/streptomycin, sodium pyruvate, Hepes buffer, and 8 μg/ml insulin (Life Technologies, Gaithersburg, MD). Aliquots of each passage from P7 to P25 were frozen in 20% serum, 10% DMSO and preserved in liquid nitrogen for future use so that different passages of the cells could be assayed at the same time under identical circumstances. The cells were determined to be mycoplasma-free by Hoechst staining. Vials of cells frozen at different passages were thawed quickly and cultured, usually through one additional passage, before they were evaluated.

Isolation of mesangial cell RNA

Cells were lysed in 4 M guanidine isothiocyanate, 25 mM sodium acetate, and 100 mM β-mercaptoethanol (Sambrook et al., 1989) and removed from the plate by scraping. After centrifugation over a $CsCl_2$ cushion (Glisin et al., 1974) overnight at 32,000 rpm, the RNA pellet was resuspended in 0.3 M sodium acetate, phenol/chloroform extracted, and precipitated with chilled, absolute ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in DEPC-treated water before quantification by absorption at 260 nm.

Northern analysis

Total cellular RNA (10 μg) was subjected to denaturing (formaldehyde) gel electrophoresis through 1% agarose in Mops buffer before overnight transfer to nylon membrane by capillary action. The RNA was crosslinked to the membrane by ultraviolet irradiation. ^{32}P-labeled cDNAs were hybridized with the blots in QuickHyb (Stratagene) at 65°C, for 1 hour. The blots were washed at serially increasing stringency, dried, and exposed to pre-flashed X-ray film at −70°C for the purposes of photography. Signal intensity was quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Studies of mRNA expression were confirmed by evaluation of at least three different preparations of RNA from cells harvested at different times; in some instances, RNA from the same preparations was subjected to electrophoresis and transfer more than once, with similar results on separately hybridized blots.

cDNA probes used in hybridization

cDNAs for human α1(I), α1(III) and α1(IV) collagen chains, and for laminin α1 (representing the carboxy-terminal end of the EHS laminin α chain), β1, and γ1 chains, were obtained from L. Bruggeman and Y. Yamada, National Institute of Dental Research, NIH. The probes for gelatinase A (MMP-2) (Collier et al., 1988) and gelatinase B (MMP-9) (Wilhelm et al., 1989) were obtained from G. Goldberg,

Washington University, St Louis, MO. The TIMP-1 cDNA (Carmichael et al., 1986) was obtained from D. Carmichael, Synergen, Boulder, CO; TIMP-3 cDNA was obtained from D. Edwards, University of Calgary. The TIMP-2 (Stetler-Stevenson et al., 1990) and interstitial collagenase (Brown et al., 1990) cDNAs were prepared as described. Probes for tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) were kindly provided by D. Dichek, NCI (Dichek et al., 1989). The cDNA for PAI-1 (Feinberg et al., 1989) was provided by E. S. Barnathan, University of Pennsylvania. The PAI-2 cDNA (Ye et al., 1987) was obtained from the American Type Culture Collection. Blots were hybridized with cDNA for 28 S (H. Sage, University of Washington, Seattle WA) or 18 S (D. Edwards) ribosomal RNA to control for loading.

Immunoblot analysis

Cells from different passages were plated at identical density in 150-mm culture dishes. Three days later, before the cells were confluent, the medium was replaced with serum-free medium. After 18 hours, the supernatant fluid was harvested and subjected to SDS-PAGE in a 10% gel under reducing conditions before electrophoretic transfer to Immobilon-P membranes (Millipore). The membranes were dried, spotted with positive controls, and blocked with BSA. They were then developed with a 1:50 dilution of sheep anti-mouse type I collagen or 1:100 dilution of rabbit anti-mouse type IV collagen or rabbit anti-mouse laminin. These antibodies were prepared by immunizing the respective animals against mouse skin type I collagen or against collagen IV (Kleinman et al., 1982) or laminin (Timpl et al., 1979) purified from the Engelbreth-Holm Swarm tumor; they have been shown to cross-react with human proteins. After washing, the blots were incubated with 1:500 antiserum against the primary antibody, conjugated to horseradish peroxidase. Finally, the signal was developed with 4-chloronaphthol/H_2O_2.

Indirect immunofluorescence for type IV collagen

Cells were plated at identical densities (6,000 per well) in an eight-well chamber slide (LabTek) for three days prior to fixation with 3.7% formaldehyde in PBS followed by permeabilization with Triton X-100 in PBS, and then stained with rabbit anti-collagen IV antiserum (1:100) followed by goat anti-rabit IgG-FITC (Vector), 1:100. Control wells were incubated with preimmune rabbit serum. Photomicrographs were taken using a Nikon Optiphot microscope and UFX-DX photographic system with identical times for all exposures.

Zymogram analysis

Serum-free, mesangial cell-conditioned media were evaluated for gelatinase activity as described previously (Schnaper et al., 1993). Fresh or frozen cell supernates were subjected to electrophoresis under non-denaturing conditions through a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. The gel was then incubated twice in 2.5% Triton X-100 for 30 minutes each time, and equilibrated in 10 mM Tris, pH 8, before incubation in 50 mM Tris containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 16 hours at 37°C (Birkedal-Hansen and Taylor, 1982), and then stained with Coomassie blue. Bands of protein with gelatinolytic activity appear as clear zones in the surrounding blue background.

RESULTS

Expression of mRNA for collagens, collagenases and TIMPs

FMC2 cells, thawed at passages 7, 10, 13 and 15, were cultured for one additional passage before total cellular RNA was harvested and subjected to electrophoresis. The blot resulting after northern transfer was hybridized with cDNAs for the α1 chains of types I, III and IV collagen. There was no significant change in the total signal for mRNA for α1(I) collagen (Fig. 1). There was, however, a difference in the pattern of expression observed, with decreased expression of the larger of the two species that were detected. A pronounced change with serial passage was seen when the same blot was stripped and re-probed for expression of both α1(III) and α1(IV). These mRNAs showed a marked increase in steady-state expression. There was a 4-fold increase in signal intensity for α1(III) from P11 to P14 and an additional doubling of intensity at P16 (corrected for loading). Similarly, there was a 2-fold increase in intensity of α1(IV) signal from P11 to P14 and further doubling to P16.

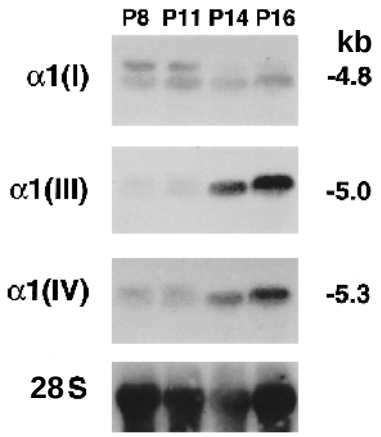

Fig. 1. Expression of mRNA for α1 chains of collagens by FMC2 cells at different passage. Cells at different passage number (P) were thawed, grown to confluence, split 1:5, and grown to approximately 85-90% confluence. Total cellular RNA was then harvested by centrifugation of cell lysates over a $CsCl_2$ cushion and subjected to denaturing electrophoresis before transfer to a nylon membrane. Equivalence of loading in different lanes was ascertained by similar intensity of ethidium bromide staining of ribosomal bands. The same blot was stripped and re-probed for expression of mRNA for the α1 chain of types I, III and IV collagen and for 28 S rRNA. Similar results were obtained in three additional experiments.

When the blots were evaluated for expression of collagenases and their inhibitors, a strikingly different pattern was observed (Fig. 2). Fibroblast-type interstitial collagenase (MMP-1) showed an intense signal at P8 that decreased 10-fold at P11, and was virtually nonexistent by P14. A strong signal for gelatinase A (MMP-2) decreased 5-fold between passages 11 and 14 (Fig. 2). There was no signal detected for gelatinase B (MMP-9) at any passage. mRNA for TIMP-1 showed a decrease similar to that seen with gelatinase A. In contrast, the total signal for TIMP-2 remained unchanged. However, there was a switch in the relative intensity of the two species of TIMP-2 mRNA, with the larger, 3.5 kb species showing 5-fold predominance at early passage and the smaller, 1.0 kb species showing 5-fold predominance by P14. These findings indicate that expression of mRNA for collagenases decreases with serial passage, opposite to the increase in expression of certain collagen mRNAs. The changes observed with collagenase inhibitors are more complex.

Changes in mRNAs related to laminin synthesis and degradation

No signal was detected at any passage for laminin α1 chain,

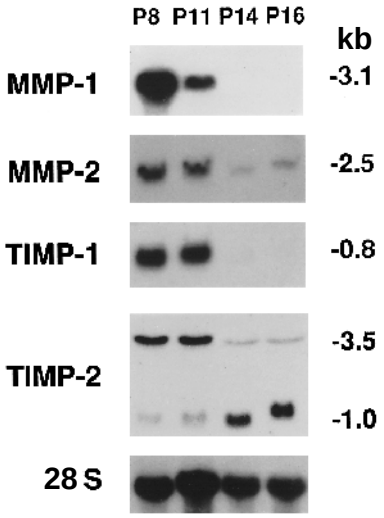

Fig. 2. Expression of mRNA for metalloproteinases and metalloproteinase inhibitors at different passages. RNA was obtained as described in the legend for Fig. 1. MMP-1, fibroblast-type interstitial collagenase; MMP-2, gelatinase A; TIMP, tissue inhibitor of metalloproteinases. A very faint signal for stromelysin-1 was detected with similar intensities at all passages, and no signal was detected for gelatinase B (MMP-9) at any passage. Similar results were obtained in 4 additional experiments.

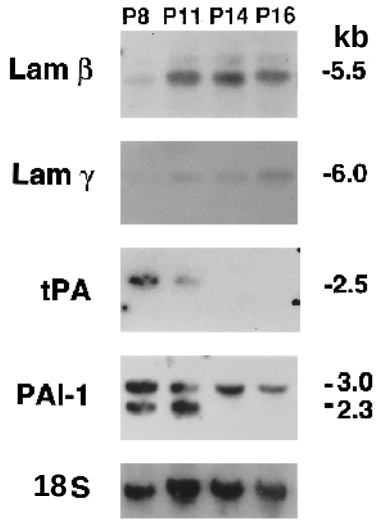

Fig. 3. Expression of mRNA for laminin and plasminogen activators. The same and similar blots to those shown in Figs 1 and 2 were stripped and re-probed for laminin α1, laminin β1, and laminin γ1, as well as for plasminogen activators and plasminogen activator inhibitors. tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1. No signal was detected for laminin α1, uPA or PAI-2.

consistent with a recent report that the laminin α chain expressed in mature, normal kidneys is distinct from the EHS laminin α (Lindblom et al., 1994) from which our cDNA was derived. However, laminin β1 showed a 4-fold increase from P8 to P11 and laminin γ1 increased 2-fold from P8 through P16 (Fig. 3). In contrast, tPA showed a 3-fold decrease from P8 to P11 and virtually disappeared thereafter. No signal was

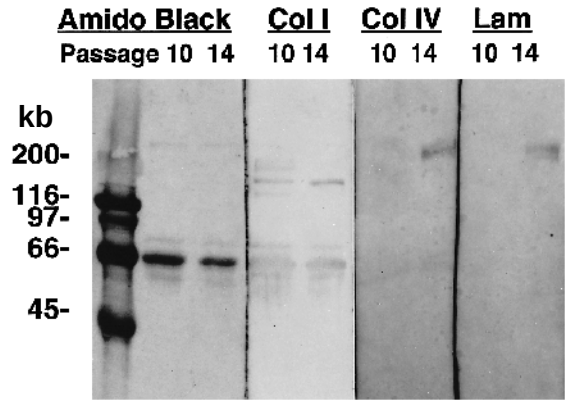

Fig. 4. Western blot for ECM proteins in medium conditioned by P10 or P14 FMC2 cells. Cultures were seeded at similar densities and grown for 3 days; at this time they appeared to have similar degrees of confluence. The culture medium was aspirated and replaced with identical amounts of medium containing no serum but otherwise identical to standard culture medium. After 24 hours, the conditioned medium was harvested and 50 μl/lane was subjected to SDS-PAGE under denaturing conditions. The supernatant fluids were run in quadruplicate pairs and each pair was stained, respectively, with amido black (to show total protein) or with antibodies to collagen I, collagen IV, or laminin. After electrophoretic transfer to Immobilon-P membranes, the blots were developed using the indicated polyclonal antisera as described in Materials and Methods. Similar results were obtained in three separate experiments.

detected for uPA at any passage. There was a faint, unchanging signal for stromelysin-1 detected at all passages (data not shown). The plasminogen activator inhibitor PAI-1 is normally expressed as two mRNA species, of 3.0 and 2.3 kb; the difference between the two transcripts is felt to reflect shorter 3′ untranslated sequence (Ny et al., 1986). Signal for the larger transcript remained essentially unchanged, with a slight decrease at P16. In contrast, the smaller transcript was not detected after P11. In general, FMC2 cells show a pattern of expression relevant to laminin turnover similar to that observed with collagen turnover, with increased expression of matrix protein and decreased expression of ECM protease mRNAs.

Production of ECM proteins by cultured FMC2 cells

Since the cells express increased amounts of mRNA for collagen IV and laminin and decreased amounts of mRNA for ECM proteases, they were examined for accumulation of ECM proteins. Media conditioned by FMC2 cells at P10 or at P14 were evaluated by immunoblot for the presence of secreted collagens and laminin (Fig. 4). Amido black staining revealed a major band of protein at about 66 kDa, probably representing residual albumin from the initial, serum-containing medium. Staining with antibody to collagen I revealed little change in expression between passages 10 and 14. There was, however, a marked increase in expression of both collagen IV and laminin. When the cells were cultured for 3 days and then stained for type IV collagen, some heterogeneity was observed (Fig. 5). However, in general the intensity of staining increased markedly as the number of passages increased. In some cases, increased punctate staining was noted extracellularly, although it is difficult to appreciate this finding from photographs due to the increased brightness of the cellular staining. These data

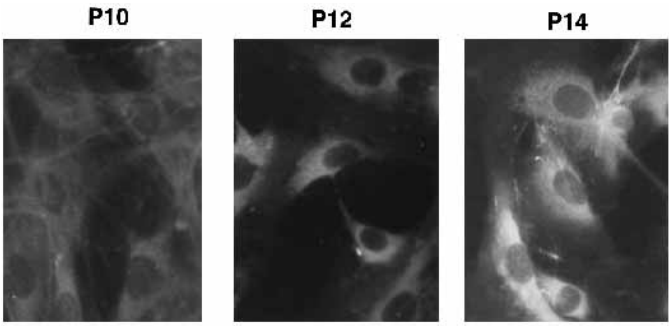

Fig. 5. Expression of type IV collagen by cells of different passage. Immunocytochemistry was performed by indirect immunofluorescence. The cells were plated on glass slides and cultured for 3 days before staining was performed as described in Materials and Methods. Representative views that include the degree of heterogeneity observed are shown in the figure. ×150.

indicate that, consistent with the northern analysis data, certain ECM proteins may accumulate in cultures of FMC2 cells subjected to an extended series of passages in culture.

Secretion of MMP activity into cell cultures

The increased amount of matrix protein could result entirely from an increase in steady-state expression of ECM protein mRNA. However, decreased ECM protease secretion might also contribute to matrix accumulation. To investigate this possibility, gelatin zymography was performed on conditioned media. Both the proenzyme (72-kDa) and activated (68-kDa) forms of gelatinase A activity were present in medium conditioned by P10 cells, but little activity was detected in medium conditioned by P14 cells (Fig. 6). Since interstitial collagenase is much less active than gelatinases in gelatin zymograms, the conditioned media were concentrated 10-fold and again subjected to zymography. Bands of gelatinolytic activity were observed at 50-55 kDa, the size of MMP-1, in P10-conditioned medium. These were not seen in P14-conditioned medium (data not shown). Thus, protease activity in the medium decreases with serial passage, consistent with changes observed in mRNA expression.

Expression of TGF-β1 mRNA by cultured cells

Because TGF-β1 has been found to play a role in several models of matrix accumulation, the cultured cells were evaluated for expression of TGF-β1 mRNA during the time at which the greatest changes in mRNA expression occur. A slight increase in TGF-β1 mRNA was seen from P8 to P14, but a much greater increase (5-fold) occurred by P16 (Fig. 7). In other experiments, the greatest increase was apparent by P14 (data not shown). These results are consistent with an increase in production of TGF-β1 with serial passage.

Effect of exogenous TGF-β1 on expression of mRNA for type I collagen, gelatinase A and TIMP-2

Since this last finding suggested that TGF-β could contribute to the phenotypic changes observed with serial passage, we tested the effects of exogenous TGF-β1 on several of the ECM components described above. Cells at P9 or P10, just before the most striking phenotypic changes occur, were cultured with graded doses of active TGF-β1 for 3 days. There was an

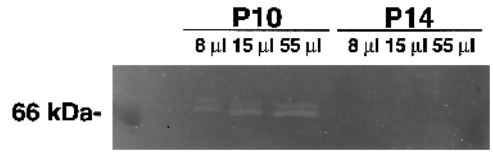

Fig. 6. Gelatin substrate gel chromatography of conditioned media from FMC2 cultures. The indicated amounts of medium were subjected to zymography as described in Materials and Methods. Although no signal was seen at any other molecular size under these conditions, when the media were concentrated 10-fold, a faint band was seen at 55 kDa with medium conditioned by P10 cells but not by P14 cells (data not shown). Similar results were obtained in 4 separate experiments.

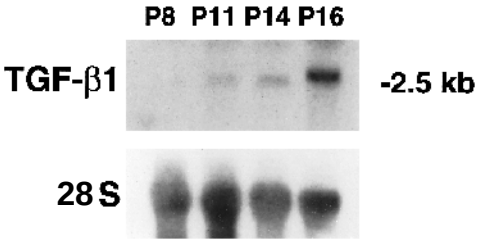

Fig. 7. Expression of mRNA for TGF-β1 at different passages. Similar results were obtained in 6 different experiments using fetal and adult cells of differing passages.

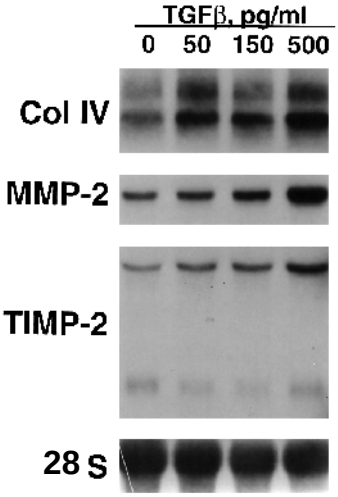

Fig. 8. Effect of TGF-β1 on expression of mRNA for collagen IV, gelatinase A and TIMP-2 mRNA. P9 FMC2 cells were treated with the indicated dose of TGF-β1 for 72 hours. To be certain that all cultures were fed and exposed to the indicated amounts of TGF-β, the medium was replaced with fresh, TGF-β-containing medium after 2 days. Total cellular RNA was harvested as described in Materials and Methods. The same blot was probed for α1(IV) collagen, gelatinase A and TIMP-2. Similar results were obtained in four additional experiments, two using cells at P9 and one each using cells at P7 and P10, as well as with mesangial cells derived from adult tissue and tested at different passages.

increase in expression of collagen α1(IV) mRNA (Fig. 8), although the extent of this increase varied in different experiments. In contrast to the findings after a prolonged series of

passages in culture, steady-state expression of mRNA for gelatinase A doubled. Moreover, TGF-β1 did not induce a change in the ratio of signal intensity comparing the two species of TIMP-2 mRNA. These results are consistent with previous reports of TGF-β-induced changes in mesangial cell mRNA expression, but are different from the changes observed in our model. In other experiments, we found that the intensity of signal for all of these mRNAs was maximal at 0.5-1.0 ng/ml TGF-β1 and decreased somewhat as the concentration of TGF-β1 approached 5 ng/ml (data not shown). Further, these results were consistently observed with cells at earlier passages as well as passages as late as P10, and with mesangial cells of either fetal or adult origin (data not shown). These findings, along with the observation that TGF-β1 mRNA increases at a later passage than does the switch in mRNAs related to ECM turnover, indicate that exogenous TGF-β1 does not cause the type of changes in cell phenotype that we have described with serial passage.

Table 1. Changes in mRNA expression with serial passage of FMC2 cells

mRNA	Change
Collagen	
α1(I)	—
α1(III)	↑↑
α1(IV)	↑↑
Laminin	
α1	Not detected
β1	↑
γ1	Slightly ↑
MMPs and TIMPs	
Interstitial collagenase (MMP-1)	↓↓↓
Gelatinase A (MMP-2)	↓↓↓
Stromelysin-1 (MMP-3)	Faint signal; no change with passage
TIMP-1	↓↓
TIMP-2	Switch in ratio of signal intensity comparing the two mRNA species
TIMP-3	Not detected
PAs and PAIs	
tPA	↓↓
uPA	Not detected
PAI-1	Decrease in smaller mRNA species
PAI-2	Not detected
TGF-β1	↑↑

DISCUSSION

Glomerulosclerosis is characterized by excessive accumulation of extracellular matrix. This process may involve deposition of abnormal matrix components, but studies have clearly indicated that additional amounts of classical components also accumulate. Thus, the net turnover of ECM in the glomerulus is altered, reflecting either increased synthesis, decreased removal, or both. Studies by various investigators have implicated both possibilities in glomerulosclerosis (reviewed by Schnaper, 1995). We subjected human fetal glomerular mesangial cells to a prolonged series of passages in culture in order to develop a model for mesangial regulation of matrix turnover. Beginning after P8, a comprehensive pattern of changes was noted, consistent with matrix accumulation (Table 1). Cells showed increased steady-state expression of mRNA for the α1 chains of types III and IV collagen and for laminin β1 and γ1. At the same or earlier passages, expression of certain ECM proteases showed a decrease. There also were marked changes in expression of two ECM protease inhibitor mRNA species, most notably a reversal of the ratio of signal intensity for the two TIMP-2 species. Concomitantly, the amount of type IV collagen and laminin present in serum-free medium conditioned by the cells for 24 hours increased, while the amount of interstitial collagenase (MMP-1) and gelatinase A (MMP-2) decreased. Cellular staining for collagen IV also increased. Thus, after a prolonged series of passages in culture, FMC2 cells manifest a matrix-accumulating phenotype, with increased synthesis and decreased degradation of ECM.

Somewhat surprising was our observation that protease inhibitor mRNA expression remained the same or even decreased with serial passage. We have not, however, excluded the possibility of post-transcriptional regulation of these proteins. Further, we have not yet addressed the importance of changes in the relative expression of the two species of TIMP-2 mRNA. The species are reported to have similar coding regions but the larger species appears to have a long 3′ untranslated region; the effect of this difference on translation into protein is the subject of ongoing investigation in several laboratories. The direct impact of the changes we have observed in protease inhibitor mRNA expression on control of ECM turnover is unclear. In the absence of significant amounts of MMPs, for example, changes in concentration of TIMPs may have little impact on proteolytic activity. However, TIMPs recently have been suggested to have additional biological roles (Murphy et al., 1993) that could affect mesangial cell function in other ways. Regardless of the significance of the changes in ECM protease inhibitor mRNA expression, these findings provides strong evidence of altered regulation of gene transcription or mRNA processing with serial passage.

The observed changes in cell phenotype could be a model for aging. In human skin fibroblasts cultured from subjects of various ages, fetal cells at P20 showed similar characteristics to earlier passages of fibroblasts from 80-year-old donors (Takeda et al., 1992). Glomerulosclerosis is a common histological finding with aging in vivo both in humans (Kaplan et al., 1975) and in animals (Couser and Stilmant, 1975). Despite this ‘aging process,’ FMC2 cells at P14 to P16 do not appear to be typically senescent in that they continue to produce proteins and show stable growth rates. Indeed, they remain viable through P25. Further, there is no general defect in mRNA or protein synthesis at P14; the fact that various mRNAs and proteins increase, remain stable or decrease in expression indicates that the cells’ synthetic machinery remains intact. The findings therefore represent specific differences between different cell passages in the regulation of expression of specific proteins. Indeed, it has been suggested that changes in patterns of matrix production represent the degree of differentiation or de-differentiation of cells (Foidart et al., 1980); our model could therefore represent an aspect of a maturation process unique to fetal (as opposed to adult) cells.

Previous studies examining other cell types have identified changes in cellular production of matrix proteins or ECM proteases with serial passage in culture that, while relevant to the present series of experiments, show significant differences. First, collagen gene expression does not invariably increase with serial passage. For example, expression of types I and III

collagen was observed to decrease with serial passage in human skin fibroblasts (Takeda et al., 1992). Second, MMP production increased with passage of retinal pigment epithelium, articular chondrocytes and corneal fibroblasts (Alexander et al., 1990; Fini and Girard, 1990; Lefebvre et al., 1991). In contrast, our experiments with FMC2 cells show stable collagen I production, increased collagen IV synthesis and decreased MMP production. The difference between our model and other reports of changes in MMP expression with serial passage are consistent with the observation that mesangial cell production of gelatinase A is regulated in a unique manner compared with other cells (Marti et al., 1994). To the extent that changes with serial passage represent a model of cell 'aging,' these differences suggest distinct aspects of the mesangial cell response to aging. Therefore, our findings could explain why the glomerulus appears to be particularly susceptible to the sclerotic process as individuals grow older.

FMC2 cells at later passages (P14 to P16 in different experiments) produced large amounts of TGF-β1 mRNA. Although it is possible that increased TGF-β1 mRNA expression does not lead to equally increased protein production, TGF-β has been associated with mesangial expansion. Therefore, we used graded doses of TGF-β1 to treat mesangial cells at a point just prior to the phenotypic switch we have observed. This treatment did cause some increase in expression of mRNA for type IV collagen, but it did not change the relative signal intensity for the larger of the TIMP-2 mRNA species and, consistent with observations of others investigating mesangial cells (Marti et al., 1994), TGF-β treatment actually increased gelatinase A mRNA expression. Thus, the phenotypic switch we have observed is not produced by adding TGF-β1 to cultures. This result is consistent with a recent preliminary report (De Heer et al., 1994) indicating that TGF-β1 production accompanies development of glomerulosclerosis but is not by itself sufficient to directly cause the lesion. However, from the present series of experiments, we cannot rule out the possibility that endogenous TGF-β (produced by the cells) participates in the switch to a matrix-accumulating phenotype.

Since cell culture was initiated from a primary isolate of glomerular mesangial cells, it is possible that two subpopulations of mesangial cells were present in the initial culture, and that one showed a decrease in growth at P8-P10 while the other grew only slowly until the same passage. This is not likely, since maintenance of a stable growth rate (calculated time for doubling of cell number in culture, 55-60 hours) within the entire culture would have required a series of remarkably coordinated changes in the growth rates of each population. Nonetheless, the possibility that the classical and matrix-accumulating phenotypes represent two different populations of cells, or that a significant minority population arose and altered regulation of matrix turnover in the rest of the cells, cannot be formally ruled out by the present series of experiments.

The difference in gene expression between earlier- and later-passage FMC2 cells has been observed six different times when the cells were subjected to serial passage. Although the cause of the observed phenotypic 'switch' is uncertain, the two phenotypes clearly define contrasting classical and matrix-accumulating populations of cultured mesangial cells. In view of the consistency with which this phenotypic switch is observed, and the fact that the pattern of differences observed is unique among published reports of such changes in serially-passaged cells, our findings suggest the potential for further insight into regulation of matrix turnover in glomerular mesangial cells from studying the FMC2 cells. Because the later-passage cells appear to be accumulating matrix, comparison of the two phenotypes may permit determination of events relevant to matrix accumulation in vivo. Thus, our findings may serve as a model for cellular events involved in the pathogenesis of glomerulosclerosis.

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