Specialized Collagen mRNA and Secreted Collagens in Human Glomerular Epithelial, Mesangial, and Tubular Cells

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ABSTRACT

Isolated glomeruli and cultured cells were examined under nonmitogenic conditions by Northern hybridization of steady-state mRNA levels for procollagens α1(I), α1(III), α2(IV), α2(IV), β-actin, and fibronectin. Procollagens concurrently secreted from these cells were characterized after limited pepsin digestion. Poly(A)-mRNA from freshly isolated whole porcine glomeruli was primarily type IV. For cultured glomerular and tubular epithelial cells, the collagen mRNA species were almost exclusively α1(IV) and α2(IV). Correspondingly, the secreted collagen was almost entirely type IV. The mRNA signals for collagens in glomerular mesangial cells included α1(I), α1(IV), α2(IV), and less α1(III). The secreted collagens were also types I and IV, with less types III and V. There were similar patterns of mRNA signal levels for the two type IV collagens and similar patterns of expression of α1(I) and α1(III). In situ hybridization showed the fibroblast and epithelial cell populations homogeneous in expressing the same mRNA signals seen by Northern hybridization. These findings establish the correlation of collagen mRNA and protein expression of collagens in differentiated glomerular cells in culture, under resting nonmitotic conditions.

Key Words: Sclerosis, Northern hybridization, cDNA, fibronectin, β-actin

METHODS

Cell Cultures

Human and porcine GEC, GMC, and cells and human skin FB were cultured from human infant post-mortem kidneys and skin, adult surgical kidney specimens (unaffected by renal cell carcinomas), and late fetal pigs. Glomerular isolation by sieving was described before (13,14), with 0.1% collagenase type CLS (Worthington Biochemical. Freehold NJ) perfusion or cortical fragment collagenase incubation before sieving. Glomeruli are usually retained on 56- to 88-μm screens for fetal and premature infant kidneys and 88 to 105 μm for infant human and porcine.
kidneys. Pure tubular preparations are obtained from the 150-µm excluded material.

GEC are cultured from whole glomeruli in K1 medium (15,16) with 5% NuSerum IV (Hyclone Labs, Bedford, MA); insulin, transferrin, and selenium, prostaglandin E1, triiodothyronine (T3), and hydrocortisone. Outgrowths are passaged within a few days of appearance, before GMC emerge. In some cultures, heparin (17,18) (100 µg/mL) and epidermal growth factor (19–21) (10 ng/mL) are initially used to selectively inhibit mesangial cell proliferation. GMC are derived from whole glomeruli denuded of epithelial cells by further incubation in 0.2% collagenase with 0.15 µg/mL of DNase. They are cultured in Waymouth’s medium (752/1) in 20% fetal calf serum, with 100 ng/mL of puromycin aminonucleoside sometimes added to inhibit residual GEC proliferation (22). TEC are cultured initially in the K1 medium described above, with 1 or 5% NuSerum IV, on collagen gels (15), and thereafter maintained on plastic.

Identification of cells is morphologic and immunohemical. GEC grow in contact-inhibited “cobblestone” monolayers. HuGEC have included one established line (studied at passages 24 to 26) that formed “domes” in long-term cultures. Most other HuGEC and porcine GEC were larger, with typically large oval nuclei, which become more elongated in later culture. TEC, otherwise similar to GEC, often maintained a whorled morphology. Immunofluorescence staining (13) of GEC or TEC show only diffuse or aggregated myosin (14). Staining is faint with antibody to γ-actin (HHF35; Enzo Biochemical, New York NY) (23). Cytokeratin monoclonal antibody (Enzo Biochemical) localizes strongly in a pattern of intermediate filaments. Surface matrix staining for type IV but not type I collagen is evident on the surface of GEC (Figure 1) but not GMC.

GMC are elongated and straplike, grow in multilayered ridges and valleys, and show strong antibody reactions of myosin fibrils (13,14) and actin fibrils (23) (Figure 2) but not cytokeratin (26). Porcine GMC are less elongated than HuGMC, as depicted by Rytter Nørgaard (27). Monoclonal antibodies to vimentin (Enzo Biochemical) (28,29) are not selective (30), staining intermediate filaments in both GMC and GEC. Antidesmin antibodies (DAKO, Santa Barbara, CA) have stained none of our cells, as is appropriate for vascular smooth muscle cells (31). Fibroblasts grow in Waymouth’s 752/1 with 20% fetal calf serum as homogeneous multilayers that reveal neither myosin nor cytokeratin markers.

In situ hybridization of 35S-labeled cDNA followed descriptions by Lawrence and Singer (32) and Lum
tained 50 μg/L of β-aminoproprionitrile and ascorbic acid for the final day. For biochemical analysis of labeled newly synthesized collagens, there was an additional overnight incubation in ascorbate/β-aminopropionitrile with [3H]proline (Amersham Corp., Arlington Heights, IL) (20 μCi/mL), 3 mL/75 mm² flask. Media for collagen analysis were removed, allowing mRNA analysis of the cell layer.

**mRNA ANALYSIS**

Cell layer RNA was extracted in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.3% sarcosyl, 0.1 M β-mercaptoethanol, and 2 M sodium acetate, as described by Chomczynski and Sacchi (36). RNA was isolated by sequential extraction in phenol–chloroform–isoamyl alcohol and was precipitated with isopropanol. A typical optical density ratio at 260/280 nm was 1.8. Cell layer (Lowry protein) quantitation used an aliquot of the initial RNA extract.

Direct analysis of endogenous steady-state mRNA level in glomeruli from piglets used rapid isolation of glomeruli at 4°C, including vanadyl-ribonucleoside complex (Bethesda Research Laboratories, Gaithersburg, MD) to inhibit RNAse activity. Poly(A+)+ RNA was isolated by spin-column oligo-(dT) (Fast Track; In Vitrogen, San Diego CA), as described by the vendor.

cDNA in plasmids were kindly provided for α(I) (HF677 [37]), α(II) [38], α(III) (HF 934 [39]), and α(V) (HF 511) [40] by Dr. Franco Ramirez; α(IV) (HT 21) and α(VI) (HD-4) from Hostikka and Tryggvason [41]; for β-actin (B668) from Gunning et al. [42]; for FN (rif-1) [43] from Schwarzhauer et al.

Hybridizations for Northern analysis were performed by the method of Maniatis et al. [44]. Equivalent amounts of total RNA were applied to lanes of agarose gels. GeneScreen-Plus (DuPont, NEN Research Products, Boston, MA) was used for agarose gel transfer and slot blot (DuPont, NEN Research Products) of RNA. During hybridization, the temperature was adjusted to maximize stringency for each cDNA/mRNA pair. There was no apparent difference in hybridization strength between human and porcine cells. Autoradiography on XAR-5 film (Eastman Kodak, Rochester, NY) was scanned by either an LKB or Tobias Associates (Ivy Land, PA) densitometer. The linear range of autoradiographic density was determined over a 10-fold range of RNA concentration on slot blots. On Northern scans, the density (expressed as areas of scans) representing the specific collagen mRNA hybridized by a single cDNA in different cells was corrected for total RNA applied and cell layer protein. Alternatively, the density was related to the corresponding peak area of β-actin or FN in the same lane of the gel.

**MEDIUM COLLAGEN ANALYSIS**

To assess the phenotype of collagen excreted into the medium protein, collagens were labelled with [3H]proline, under the experimental conditions for mRNA analysis described above. Media were harvested when the (same) cells were removed for mRNA quantitation. The radioactively labeled media of cultures were processed as described previously for cultured rat GMC [12]. Briefly, the labeled medium was collected and the cell layer was washed twice with proteolytic inhibitors n-ethylmaleimide (10 mM) and phenylmethylsulfonyl fluoride (0.5 mM). The sample was kept at -70°C before fractionation of the collagen proteins. The medium was initially precipitated at 30% saturated ammonium sulfate, suspended, and dialyzed versus 0.5 M acetic acid and digested at 100 μg/mL of pepsin at 4°C for 24 h. The collagen was precipitated at 1.2 M NaCl, redissolved in 1 M NaCl (pH 8), precipitated at 4.5 M NaCl, and dissolved in 0.5 M acetic acid. Both the interstitial (types I and III) collagens, precipitated at 0.9 M NaCl, and the supernatant, usually types IV and V collagen, were examined by PAGE under reducing and nonreducing conditions.

Selected specimens were subjected to agarose chromatography after reduction and alklylation, to separate α-chain fractions. The fractionation of α-chains for collagen phenotype employed CM-Trisacryl (Pharmacia LKB, Piscataway, NJ) chromatography in 20 mM sodium acetate and 1 M urea at pH 4.8 (42°C), and a linear gradient from 15 to 90 mM NaCl.

**RESULTS**

**mRNA Analysis in Glomeruli and Cell Cultures**

Poly(A) RNA from freshly isolated whole porcine glomeruli was subjected to dot-blot hybridization at 25 to 200 ng/spot. Autoradiographic density was eightfold greater with α(I) [slope 4.3 U/ng] than with α(I) [slope 0.54]. In comparison, mRNA concurrently isolated and hybridized from FB showed the opposite pattern with α(I) [slope 3.0] versus slope 0.17 for α(I).

Total RNA from human FB, GEC, and GMC, and porcine GEC, TEC, and GCM, was isolated and demonstrably (by agarose gel estimation) preserved without evidence of degradation. An initial experiment compared steady-state mRNA levels by slot-blot analysis with agarose-purified cDNA inserts. The α(I) cDNA and α(I) cDNA hybridized with greatest density to the RNA from the FB lines. α(IV) and α(V) had greater density with GEC and GMC lines. As estimated by both α(IV) probes, α(IV) mRNAs made up a relatively insignificant contribution to the FB collagen mRNA pool. There was no clear difference in mRNA patterns between cells maintained at con-
TABLE 1. Northern hybridization of cell culture mRNA

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>cDNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-actin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α&lt;sub&gt;1&lt;/sub&gt; (I)</td>
<td>α&lt;sub&gt;2&lt;/sub&gt; (I)</td>
<td>α&lt;sub&gt;1&lt;/sub&gt; (III)</td>
</tr>
<tr>
<td>HuFB-Long</td>
<td>0.92</td>
<td>1.57</td>
<td>0.60</td>
</tr>
<tr>
<td>HuFB-Short</td>
<td>0.53</td>
<td>2.14</td>
<td>0.84</td>
</tr>
<tr>
<td>HuGEC-Long</td>
<td>0</td>
<td>0.26</td>
<td>0.34</td>
</tr>
<tr>
<td>HuGEC-Short</td>
<td>0</td>
<td>0.62</td>
<td>0.46</td>
</tr>
<tr>
<td>Porcine GEC-Short</td>
<td>0</td>
<td>0.70</td>
<td>0.36</td>
</tr>
<tr>
<td>Porcine Tub-Short</td>
<td>0</td>
<td>3.78</td>
<td>1.83</td>
</tr>
<tr>
<td>HuGMC-Long</td>
<td>1.30</td>
<td>4.57</td>
<td>0.19</td>
</tr>
<tr>
<td>HuGMC-Short</td>
<td>0.49</td>
<td>1.39</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup> Area of scanned peak as autoradiographic density, per milligrams of cell protein x 10<sup>-3</sup>. Values were corrected for the amount of total RNA applied to the gel.

<sup>b</sup> Sample SD; N = 4.

Figure 3. Northern hybridization of agarose-separated cell culture RNA, with 32P-labeled cDNA probes: RNA samples were first hybridized with a collagen cDNA probe alone (right panels), and then same filter was hybridized with the cDNAs for β-actin and FN (left panels). The collagen cDNAs illustrated here are α<sub>1</sub>(IV)(a) and α<sub>1</sub>(I)(b).

Figure 4. Graphic depiction of relative collagen mRNA densities for the different cell culture lines. Each cell RNA sample was hybridized with the different labeled collagen cDNAs and simultaneously with the β-actin and cDNA. (See Figure 3 for autoradiography data from which this was derived.)

fluency for short and long periods. Northern hybridization of selected samples confirmed that α<sub>1</sub>(I) and α<sub>1</sub>(IV) cDNA slot blots accurately reflect the exclusive mRNA species.

The second set of experiments used agarose electrophoresis and Northern gel hybridization of all RNA samples, directly comparing FB, GEC, TEC, and GMC under short or long confluency conditions (Table 1). RNA was hybridized to α<sub>1</sub>(I) (Figure 3b), α<sub>2</sub>(I), α<sub>1</sub>(III), α<sub>1</sub>(IV) (Figure 3a), and α<sub>2</sub>(IV) cDNA species, alone (Figure 3a and b, right panels) and together with β-

actin and fibronectin cDNA probes (Figure 3a and b, left panels). Results were visualized as a ratio of densities for cells hybridized simultaneously for the same probe (Figure 4). For all cDNAs, hybridization density was greater for human FB and HuGMC than for HuGEC lines, whether relative to cell protein (Table 1) or as a proportion of total collagen hybridization density (not illustrated). As a ratio to β-actin, the α<sub>1</sub>(I) collagen mRNAs' density was most prominent in FB and GMC and insignificant in GEC and TEC. Type III collagen was only prominent in FB, although measurable in GMC.

The type IV collagen cDNA probes showed the most density with GMC, GEC, and TEC cell RNA (Table 1; Figure 4), compared with FB, which had the least α<sub>1</sub>(IV) and absent α<sub>2</sub>(IV). In the glomerular cells, when the collagen mRNA density was related to either cell layer protein level or to β-actin, the hybridization patterns for α<sub>1</sub>(IV) and α<sub>2</sub>(IV) collagens are similar to
each other. Likewise, the $\alpha_1(\text{I})$ and $\alpha_1(\text{III})$ species are similar in the GMC and FB.

The time that calls were maintained in nonmitogenic conditions, short (1 to 2 days) versus long (6 days), before harvest showed no consistent effect on the cell mRNA patterns, although there is a trend to increased type I collagen mRNA in FB and GMC and increased type IV in GMC. Porcine GEC and HuGEC were similar. Porcine GMC were not directly compared in this experiment. Significant $\alpha_2(\text{I})$ density, besides its expected appearance with $\alpha_1(\text{I})$ in FB and GMC, was found in the porcine TEC and GEC lines in which $\alpha_1(\text{I})$ was not found.

*In situ* hybridization of $^{35}$S-labeled cDNA to HuFB-long and HuGEC-long showed distinctive and homogeneous localization: FB had a strong autoradiographic signal for $\alpha_1(\text{I})$ and a minimal one for $\alpha_1(\text{IV})$. GEC demonstrated the reverse: strong $\alpha_1(\text{IV})$ but not $\alpha_1(\text{I})$ (Figure 5).

**Medium Collagen Secretion**

Salt fractionation of pepsin-resistant collagens resulted in most of the labeled material precipitating in the interstitial (types I and III) fraction, at 0.9 M NaCl. PAGE analysis (Figure 6a) showed that in the FB and GMC samples, the predominant components corresponded to the mobility of the type I collagen chains, $\alpha_1(\text{I})$ and $\alpha_2(\text{I})$, in nonsulfide-bonded molecules. A lesser amount of material migrated more slowly in unreduced gels (Figure 6b) and disappeared on reduced gels (Figure 6a), corresponding to $\alpha_1(\text{III})_3$ molecules.

For the GEC and TEC samples, the predominant collagen species from the 0.9 M (expected types I and III) fraction migrated with the mobility of 140-kd and 100-kd type IV collagen chains (Figure 6a). This was due to (anomalous) precipitation of types IV and V in this interstitial fraction. As is especially visible on these reduced gels (Figure 7a), there was only trace material corresponding to $\alpha_1(\text{I})$ collagen or $\alpha_1(\text{III})$ chains.

In the 0.9 M NaCl supernatant, the types IV and V fraction, from GEC and TEC (Figure 6c), collagens migrated on PAGE in patterns similar to the collagens precipitated in the types I and III fraction, as $\alpha_1(\text{IV})$ and $\alpha_2(\text{IV})$. The small amount of collagen from FB that appeared in the types IV and V fraction migrated with the mobility of $\alpha_1\alpha_2(\text{V})$ chains. The predominant

![Figure 5](image_url)

**Figure 5.** *In situ* hybridization with $^{35}$S-labeled cDNA for $\alpha_1(\text{IV})$ (a and b, both magnifications, x40) or $\alpha_1(\text{I})$ (c, magnification, x25, d, magnification, x40) on HuFB (a and c) and HuGEC (b and d). Type IV collagen mRNA is seen only in GEC, whereas Type I collagen mRNA is seen only in FB.
collagen in the interstitial fraction from GEC and TEC was therefore type IV, with little types I, III, or V collagens.

In GMC samples, fractionation of collagens by salt precipitation appropriately separated the types I and III and types IV and V fractions, showing almost all types I and III material to be type I collagen and least type III (Figure 6b). The types IV and V fraction from GMC was predominantly type IV, but there was clearly significant type V collagen (Figure 6c). Agarose chromatography performed on four representative samples (HuFB-long, HuGEC-long, HuGMC-long, HuGMC-short) separated the α-chains, with 32 to 68% recovery. By CM-Trisacryl gradient chromatography of the α-chain collagens, the patterns of medium collagen secretion (Figure 7) were similar to the mRNA patterns for these cells (Figure 4). In addition, analysis of the types I and III fraction of FB and GMC cell media showed greater than the expected twofold excess of α1(I) chains over α2(I) chains, and therefore the presence of α1(I)3 homotrimer.

**DISCUSSION**

These studies have shown that accumulated mRNA levels for the collagen chains in FB and glomerular cells are appropriately associated with simultaneous medium collagen secretion of those chains. GEC and TEC almost exclusively transcribed the basement membrane types α1(IV) and α2(IV) collagen mRNA and secreted those collagen chains. The type IV chains appeared similarly regulated, as is reasonable for genes with a shared promoter (45). FB contained almost exclusively interstitial [Types α(I) and α1(III)] collagen mRNA and secreted those collagen chains. In situ hybridization strengthened the likelihood that these cells were homogeneous.

GMC exhibited both interstitial [α(I), α1(III)] and basement membrane [types α1(IV) and α2(IV)] collagen chains and mRNA. In addition, analysis of secreted collagens demonstrated smaller amounts of the α(V) collagen chains in FB and GMC. Immunofluorescence showed both type I and type IV collagens homogene-
ously distributed in the extracellular matrix over the GMC. Thus, cell type heterogeneity does not explain collagen heterogeneity. Generally, the patterns of medium collagen secretion were similar to the mRNA patterns for these cells. It is therefore likely that collagen steady-state mRNA level controls the level of resultant collagen phenotype.

These data, observed in cells removed from a proliferative environment, are compatible with our previous analyses by PAGE (5) and those of Ardaliou et al. (10) with immunoochemical collagen quantitation. In their studies, the proportion of type I collagen in GMC also exceeded by threefold that of type IV in medium analysis. In the cell layer, however, the proportion of type IV collagen was greater by twofold than that of type I. The difference between medium and cell layer-deposited collagens may explain why our mRNA signals do not show a vast excess of type I over type IV and that phenotypic alteration of our GMC may be less extreme than is apparent from the secreted product.

The earlier studies by Striker et al. (11) also showed, by PAGE and by estimates from proline and lysine hydroxylation, that GMC in the presence of PDS, synthesize both types I and IV, but the Type I was present less than in smooth muscle cells. Medium and cell layer collagen phenotypes were not separately described. The studies by Foldart et al. (9) on rat GMC estimated that secreted collagen consisted of 70% type I collagen and 15% types IV and III. Studies by Haralson et al. (12) showed almost entirely type I collagen in rat GMC, under more mitogenic and higher glucose cell culture conditions.

Species differences in collagen regulation are unlikely to explain differences in the proportions of interstitial and basement membrane collagens found in porcine GMC. Hybridization efficiency is likely similar: the (porcine) GEC and TEC in these experiments showed greater hybridization for type IV than did the (species-correct, human) GEC.

The comparison of levels of different mRNAs in the same specimens, such as \(a_2(I)\) to \(\beta\)-actin, is valid as a ratio expressing the relative effort of the cell DNA machinery toward different tasks, compared with the mRNA of other cells simultaneously hybridized. The relative pattern of a single collagen mRNA in a group of cells is compared (e.g., Figure 5), but not different collagen mRNAs in the same cell sample. The use of the constitutive mRNA, such as \(\beta\)-actin or FN, as a reference for the collagens, is used to examine the same cell type under different conditions. For example, the patterns of collagen mRNAs for HuGMC under long and short conditions are similar when related to \(\beta\)-actin. However, these constitutive mRNAs may not be regulated identically in different cell types, with shape changes reflected in \(\beta\)-actin and secretory functions reflected by FN.

The appearance of large amounts of \(a_2(I)\) mRNA in epithelial cells is puzzling. A similar observation was made by Meyers et al. (46), in which sprout bovine endothelial cells demonstrated \(a_2(I)\) without \(a_1(I)\), whereas differentiated (human) cells have usually had almost exclusively type IV collagens. The absence of the collagen phenotype of \(a_2(I)\) in GEC or TEC (by PAGE), makes \(a_2(I)\) collagen less likely to represent an important feature of their expressed collagen profile. \(a_2(I)\) in TEC could reflect a cross-hybridization with that of another collagen, although the \(a_2(I)\) cDNA apparently hybridized to appropriately sized mRNA species on the gels. Although \(a_2(V)\) would be most similar (39), this has not been fully examined. It is clear that other epithelial cells, derived from renal tubules, can secrete type I collagen (47), although a minority of collagen expressed. It is possible that type I collagen expression by epithelial cells can participate in glomerular, as well as tubular (48), sclerotic processes.

The effect of the 6-day compared with the 2-day period of quiescence was not consistent. It is therefore likely that the maximum effect of removal of serum and mitogenic stimuli was seen in the shorter time.

The analysis of secreted collagens should reflect in situ phenotype in fully differentiated cells. FB, with only interstitial collagens, and GEC, with almost exclusively Type IV collagen, clearly do this. The excessive ratio of \(a_1(I)\) chains over \(a_2(I)\) chains in those cells suggests the presence of some \(a_1(I)\) homotrimer. The type IV collagen chains secreted by GEC and GMC showed only slightly greater than a twofold excess of \(a_1(I)\) chains over \(a_2(I)\).

The large amounts of collagen mRNAs and interstitial collagens in GMC admittedly reflect an in situ phenotypic alteration, because glomeruli showed \(a_1(I)\) mRNA signal far exceeding \(a_1(I)\). This does not necessarily mimic the process of glomerular sclerosis in situ, in which interstitial collagens are minimally expressed (2). The proportions of \(a_1(I)\) found here in GMC are still apparently less than those in prior studies of rat GMC. It is unlikely that mitogens are still present in our media to explain GMC interstitial collagen mRNA and product (49). Although GMC collagen may be increased by interleukin-1 (34), in other situations, interleukin-1 decreases collagen synthesis (50). The full differentiaton of the GMC may depend upon the associated (collagen type I, inhibitory) presence of other cells, either GEC or even the glomerular endothelial cell. If the GMC is otherwise similar to the vascular smooth muscle cell, the cellular environment may be a vital differentiating factor, not present in vitro. Studies in this and other laboratories (51,52) have begun to examine the influence of cultured cells on each other in vitro.

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