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Modulation of collagen synthesis in human glomerular epithelial cells by interleukin 1

I. TORBOHM, B. BERGER, M. SCHÖNERMARK, J. VON KEMPIS, K. ROTHER & G. M. HÄNSCH
Institut für Immunologie, der Universität Heidelberg

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SUMMARY

Human glomerular epithelial cells produce matrix material e.g. collagen type IV. *In vitro*, the synthesis of collagen can be monitored by the incorporation of ^{3}H-proline, a precursor molecule of the collagens. We report on the enhancement of collagen synthesis by glomerular epithelial cells with highly purified or recombinant Il-1. Since Il-1 is released from monocytes or glomerular mesangial cells by inflammatory mediators, our results point to a participation of Il-1 in the development of sclerosis, which is seen in many forms of chronic inflammatory diseases.

Keywords collagen synthesis glomerular epithelial cells interleukin 1

INTRODUCTION

Progressive glomerular sclerosis is a hallmark of many forms of glomerulonephritis (Glassock *et al.*, 1981). Sclerosis results in part from excessive production of extracellular matrix; deposits of collagen type IV have been found in areas of mesangial sclerosis and membranoproliferative nephritis, deposits of collagen type III in the renal interstitium (Striker *et al.*, 1984). Various factors affecting glomerular sclerosis have been identified. Sclerosis can occur as a consequence of autoimmune reactions, hypertension or hyperfiltration (Cotran, 1982; Brenner, 1983). Factors directly responsible for the production of sclerotic material, however, have not been recognized yet. From in-vitro study there is evidence that monocyte products might modulate the production of collagens by glomerular cells (Killen *et al.*, 1982), which most probably exclusively produce the sclerotic material (Adler *et al.*, 1986). We reported previously on the effect of the terminal complement components C5b-9. In sublytic doses they stimulated the type IV collagen synthesis in human glomerular epithelial cells (Hänsch *et al.*, 1987). The finding might explain earlier data by Adler *et al.*, (1986), who described, in the experimental membranous nephropathy of the rat, an association of C5b-9-neoantigens with the sclerotic areas.

In the present study we tested the effect of another inflammatory mediator, namely interleukin 1 (Il-1), since Il-1, aside from many other biological activities, also affects the collagen synthesis in a number of cells. Enhancement of collagen synthesis has been reported (Melcion *et al.*, 1982; Krane *et al.*, 1985; Matsushima *et al.*, 1985; Kähäri *et al.*, 1987), but also inhibition (Pujol et al., 1985; Bhatnagar *et al.*, 1986). With regard to the development of sclerosis in the glomerulum, modulation of collagen synthesis by Il-1 is of special interest, because Il-1 is not only produced by cells of monocyte/macrophage lineage, but also by glomerular mesangial cells, GMC (Lovett et al., 1983); as in monocytes/macrophages, the synthesis of Il-1 is increased after stimulation of the GMC by lipopolysaccharide (LPS) or by the terminal complement components (Lovett *et al.*, 1987). We found that Il-1, as well as Il-1 containing monocyte supernatants, increased the synthesis of collagen, especially that of type IV.

Correspondence: Gertrud M. Hänsch, Institut für Immunologie der Univ. Heidelberg, Im Neuenheimer Feld 305, 6900 Heidelberg/FRG.

MATERIALS AND METHODS

Human glomerular epithelial cultures (GEC)
GEC were cultivated from outgrowth cultures of human glomeruli. Glomeruli were isolated from kidneys obtained from the Surgical Hospital (University of Heidelberg), mostly from patients with renal tumours undergoing nephrectomy. The cortex from tumour-free tissue was minced and the glomeruli were isolated by sieving as described by Krakower & Greenspon (1954).

The glomeruli were washed repeatedly in Hanks' balanced salt solution (HBSS) and suspended in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated fetal calf serum, 10% vitamin mix (×100) and antibiotic/antimycotic 1% (all obtained from Gibco, Eggenstein, FRG) at 300 glomeruli/ml. The glomeruli, seeded in tissue culture plates (Primaria, Falcon, obtained through Becton and Dickinson, Heidelberg, FRG), were incubated at 37 °C in a moist atmosphere containing 5% CO_2 in air. After 3 days non-adherent glomeruli were removed by rinsing with medium. After another 2 days, when cells began to sprout around the

glomeruli, the glomeruli were removed by washing vigorously in medium. The cells were cultured further until they reached confluence, which occurred between day 10 and 15.

Subcultures of GEC

Confluent cell layers were removed with trypsin-EDTA (Biochrom, W-Berlin). The cells were suspended in undiluted FCS, washed repeatedly and seeded into tissue cultured plates (1×10^4) in the medium described above. The subcultured cells reached confluence after 3–5 days. GEC were subcultured repeatedly (up to four times) without changes in morphology (judged by phase contrast microscopy) or in the ability to produce collagen in response to C5b-9.

Characterization of the cultured cells

Essentially the criteria proposed by Striker & Striker (1985) were used. The confluent cells appeared morphologically homogenous. They were characterized further by cytoskeletal staining. Here the cells were sub-cultivated on collagen-coated coverslips. Subcultures were obtained by removing the cells by trypsin-EDTA (Biochrom, W.-Berlin) according to the instructions of the supplier, washed in the culture medium, and placed on cover slips at a density of 2×10^4/ml. The cover slips were placed into Quadriperm plates (Heraeus, Hanau, FRG) and the cells were allowed to grow for another 5 days; by that time, they had reached near confluence. For indirect immunofluorescence staining, the cells were fixed with methanol (96%) at −20°C (5 min), followed by acetone (−20°C, 15 min). After washing with phosphate-buffered saline (0·01 M pH 7·4), the fixed cells were incubated with 20% normal rabbit serum, then the anti-cytokeratine-antibody, anti-PAN-Cytokeratin (Boehringer, Mannheim, FRG) was added in a final concentration of 4 μg/ml. After overnight incubation (4°C) and several washings in buffer, the cells were incubated with FITC-conjugated rabbit anti-mouse IgG (Dianova, Hamburg, FRG) in a 1:20 dilution for 2 h at room temperature. Following extensive washing, the cells were mounted in 50% glycerol and examined. A positive staining with PAN-cytokeratin was seen in GEC.

Collagen synthesis

Collagen synthesis of epithelial cells was measured in the primary cultures when cells had reached near confluence. Synthesis was always measured in six parallel cultures obtained from one donor. All experiments were reproduced with cells from at least four different donors. Altogether cells from 18 different donors were tested. *De novo* collagen synthesis was measured by the incorporation of ^{3}H-proline into the collagenase-digestible material, modified after the method described by Peterkofsky & Diegelmann (1971) and Killen & Striker (1979). Cells were incubated for 2 h in serum-free minimal essential medium with Hanks' salt (MEM) (Serva, Heidelberg, FRG) containing 0·1 M each of L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-serine (all amino acids obtained from Serva, Heidelberg, FRG) 1% vitamin mix and 1% antibiotics in the presence of β-amino-proprionitrile and Na-ascorbate, (50/μg/ml each) (Sigma, Deisenhofen, FRG). For stimulation Il-1 or medium was added respectively. The cells were incubated with ^{3}H-proline (spec. activity 900 mCi/mg, concentration 1·0 mCi/ml, Amersham Buchler, Braunschweig, FRG) in a concentration of 20/μCi/ml/well. After 24 h incubation at 37°C, supernatants of six wells were pooled. To the supernatant, EDTA was added to a final concentration of 10 mM. The supernatants were concentrated and dialysed using a Centricon 30 microconcentrator (Amicon, Witten, FRG). After five dialysis-concentration steps, free ^{3}H-proline was no longer detectable in the concentrates; also the filtrate showed only negligible amounts of radioactivity.

Collagenase digestion

Collagenase (CLSPA Worthington, purchased from Cooper Biomedicals, München, FRG), 100 units, in a total volume of 1 ml 0·05 M Hepes-buffer without EDTA, was added to the filtrate (500/μl). After incubation for 60 h at 37°C, again concentration and dialysis in Centricon devices were performed. Aliquots of the filtrates and the concentrates were counted in the β-counter or applied to SDS-PAGE, respectively.

SDS-PAGE, Western-blotting and autoradiography

Polyacrylamide gradient gels (4–17%) in a Laemmli system were used (Laemmli, 1970). Of the concentrates, 50 μl were applied after boiling with sample buffer, containing 5% 2-mercapto-ethanol (Biorad, München, FRG). As molecular weight markers, gelatin monomers (95 kD), dimers (190 kD) and trimers (285 kD) (Serva, Heidelberg, FRG) were used. After the run the proteins were transferred to nitrocellulose (Schleicher und Schüll, Dassel, FRG) and stained with Ponceau red (Sigma Diagnostics, Deisenhofen, FRG) diluted 1:2 in water. From each lane strips were cut, covered with Tris-buffered saline pH 7·4 (TBS) containing 2% casein. A monoclonal antibody against human type IV collagen (PHM 12, Australian Monoclonal Development, provided by Sebak, Aidenbach, FRG) was used in a final concentration of 1:100. As second antibody, peroxidase-conjugated anti-mouse IgG (Dianova, Hamburg) was used in final concentration of 1:5000. Bands were developed as described above. For autoradiography the SDS-gels were soaked 45 min in Enlightning rapid autoradiography enhancer (NEN, Dreieich, FRG) and dried afterwards. The films (Kodak, XAR5) were exposed to the gels for 5 to 10 days.

Quantification of collagen synthesis

Various methods were used for quantification. To measure overall synthesis, radioactivity associated with the collagenase-digestible material, obtained after removal of free ^{3}H-proline, was counted. To compare the relative increase in collagen synthesis, the autoradiographs were scanned in a Hitachi Scanner and the areas under the peaks were quantified. By this method the relative increase in ^{3}H-proline incorporation into individual bands could be determined. Furthermore, after autoradiography, the respective bands were sliced from the dried gel and after suspension in Unisolve (Zinsser, Frankfurt, FRG) counted in a β-counter. Since, using antibodies, only the 180 kD protein could be positively identified as type IV collagen, incorporation of radioactivity into the 180 kD was used for quantification of type IV synthesis.

Interleukin 1

Human monocytes (2×10^6/ml) were stimulated with LPS (S-typhimurium, Sigma, München, 10 μg/ml) for 24 h. Culture supernatant was collected and Il-1 was purified as described by Kronheim *et al.* (1985). This procedure yielded a preparation that gave a single band in a silver-stained SDS-PAGE (PHAST-System, Pharmacia). The activity was determined in a thymo-

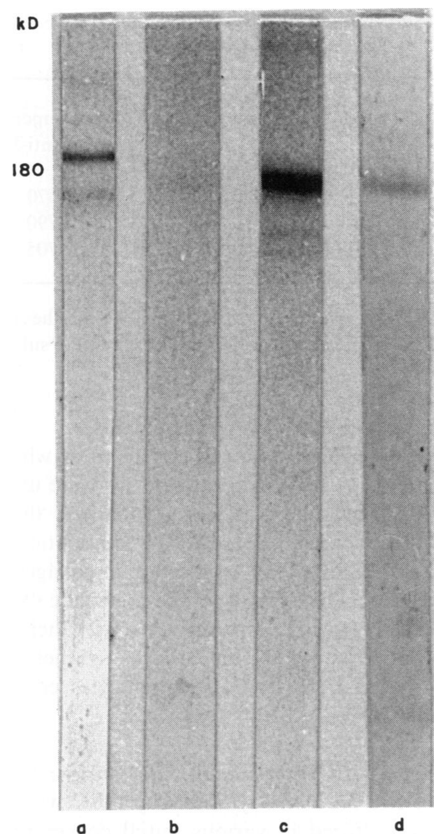

Fig. 1. Induction of collagen synthesis in GEC. GEC were cultivated in the presence of ^{3}H-proline. After 24 h the supernatants were dialysed, concentrated and submitted to SDS-PAGE. The autoradiograph showed a band in the area of 180 kD (a) which disappeared after treatment with collagenase (b). When the cells were incubated with IL-1 (1 unit) for 24 h, the intensity of the 180 kD band was drastically enhanced (c). By immunoblotting with anti-type IV collagen, the 180 kD band was identified as type IV collagen (d).

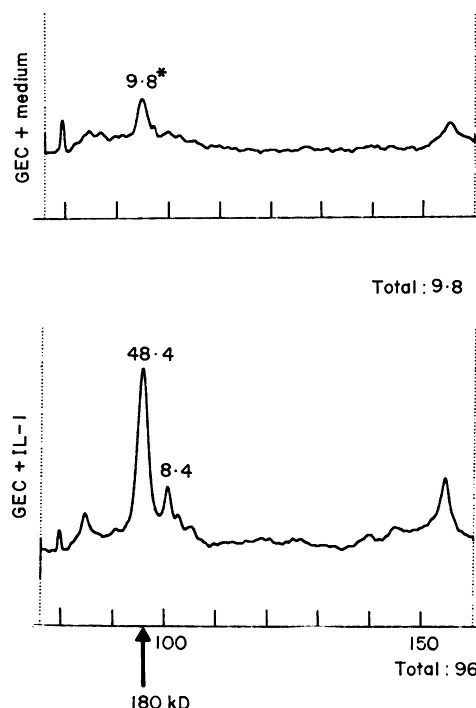

Fig. 2. Quantification of collagen synthesis. By scanning the relative amount of the synthesized collagen was determined. The numbers (*) indicate the area under the respective peaks. With 5 units IL-1, a 10-fold increase in collagenase-digestible material was seen, a five-fold increase in the 180 kD band.

cyte-proliferation assay. A unit was defined as the amount causing a doubling in cell growth. For control purposes, recombinant Il-1 and ultrapure-Il-1 produced by Genzyme and purchased through ICN Chemicals, München, were used. Furthermore, unseparated culture supernatants of LPS-stimulated monocytes were used as source of Il-1.

Quantification of Il-1

Il-1 was quantified using a radioimmunoassay obtained from Cistron Biotechnology, Pine Brook, NJ. Its functional activity was determined in a thymocyte proliferation assay modified according to Kronheim *et al.* (1985). The specificity of the assay was controlled with a polyclonal antibody directed against Il-1. The antibody (Genzyme, ICN Chemicals, München) abolished the proliferative response.

Determination of DNA synthesis by GEC

GEC were subcultured and seeded in various concentrations ranging from 10^3 to 10^5 in microtitre plates. After allowing growth for various times, the cells were incubated with ^{3}H-thymidine for 72 h. Incorporation of radioactivity into the cell was measured.

Table 1.

Kidney donor	Basal production*	After stimulation with Il-1 (1 u)
I	1638	25 107
II	840	4089
III	3670	57 491
IV	1575	31 740

Results are expressed as ct/min.

* Measured as incorporation of ^{3}H-proline into the collagenase-digestible material.

RESULTS

Effect of interleukin-1 on collagen synthesis

GEC as outgrowth cultures of human glomeruli were incubated with highly purified Il-1 for 24 h in the presence of ^{3}H-proline, aminoisoproprionitrile and sodium ascorbate to prevent cross-linking. After the incubation the supernatants were dialysed extensively, separated by SDS-PAGE and autoradiographed. GEC in culture produced some radioactive-labelled protein which appeared in the SDS-PAGE as a 180 kD protein (Fig. 1).

Il-1 enhanced the synthesis

By scanning of the autoradiograph, a considerable increase of radiolabelled proteins was seen (Fig. 2). A five-fold increase in

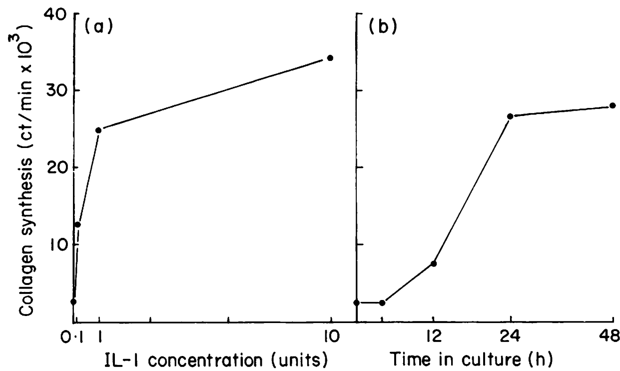

Fig. 3. Stimulation of collagen type IV synthesis in GEC incubated with IL-1. When measured after 24 h, a dose-dependency in the synthesis, measured as incorporation of ^{3}H-proline into the collagenase-digestible material, was seen (a). With 1 unit IL-1, increase in synthesis was seen after 12 h, a plateau was reached after 24 h (b).

Table 2. Stimulation of collagen synthesis in GEC by various sources of Il-1.

GEC stimulated with	Collagen production (ct/min)
Unseparated monocyte supernatants	8432±314
Purified Il-1	7483±899
Commercially available purified Il-1	5718±745
Recombinant Il-1	4015±612
Unstimulated GEC	1478±234

Collagen production measured as incorporation of ^{3}H-proline into the 180 kD protein.

the 180 kD range was seen with large Il-1 doses; additional bands appeared at 170 and 165 kD, at 62 kD and 23 kD. The ^{3}H-labelled proteins were susceptible to digestion with collagenase (Table 1). In a Western Blot with antibodies directed against collagen type IV, a staining of the 180 kD band was seen (Fig. 1), indicating that the radiolabelled-protein consisted mainly of collagen type IV.

The IL-1 effect was dose-dependent

With increasing amounts of Il-1 an increase up to 20-fold in collagen type IV synthesis, measured by quantifying the peaks under the 180 kD protein (Fig. 3), was obtained. Incorporation of radioactivity was seen first 12 h after exposure to Il-1 and progressed up to 48 h (Fig. 3). The response of the GEC to Il-1 obtained from kidneys of individual donors varied slightly, but with all cell cultures an increase in collagen synthesis was seen (Table 1). For control purposes, commercially available ultrapure IL-1 was used as well as recombinant Il-1. The ultrapure IL-1 and the recombinant Il-1 produced a 4-fold and 2·7-fold increase in collagen type IV synthesis, respectively, measured by counting the radioactivity associated with the 180 kD band (Table 2). The smaller stimulatory capacity of the two Il-1 preparations probably reflects their lower functional activity, since they were also less active in stimulating thymocyte proliferation, compared on the basis of functional units given by the suppliers.

Table 3. Stimulation of collagen synthesis in GEC by monocyte supernatants

Monocytes stimulated with	Collagen production* (ct/min)	Collagen supernatants plus anti-Il-1
C5b-9	14 300	3470
LPS	23 370	14 790
—	8740	705

* Measured as incorporation of ^{3}H-proline into the collagen-digestible material; values for the basal release were subtracted.

Induction of collagen by monocyte-supernatants

Enhancement of collagen synthesis was also seen when, instead of purified Il-1, supernatants of monocytes were used. Supernatants of unstimulated monocytes enhanced the collagen synthesis only moderately, whereas after stimulation of monocytes by either LPS or C5b-9 an increase in collagen synthesis (Table 3) was seen. The capacity of the monocyte supernatants to induce the collagen synthesis correlated with their content of Il-1. With antibodies to Il-1, the collagen synthesis by supernatants of treated LPS- or C5b-9-monocytes could be suppressed (Table 3).

Determination of GEC proliferation in the presence of Il-1

The effect of Il-1 on DNA synthesis by GEC was examined. Cells were subcultured in various initial concentrations and allowed to grow for 1–5 days. Then incorporation of ^{3}H-thymidine was measured. A doubling of cells was seen after 2–4 days. Il-1 was not found to stimulate DNA synthesis or cell growth by GEC at concentrations which increased the rate of collagen synthesis (results not shown).

DISCUSSION

The effect of Il-1 on collagen synthesis by human glomerular epithelial cells was measured by incorporation of the precursor ^{3}H-proline. After culture in the presence of aminoisoproprionitrile and Na-ascorbate to prevent cross-linking (Peterkofsky & Diegelmann, 1971; Killen *et al.*, 1982), collagen was measured in the supernatant after removal of free ^{3}H-proline. GEC produced one major radiolabelled protein with an apparent molecular weight of 180 kD in SDS-PAGE with gelatin polymers used as molecular weight markers. When globular proteins were used as marker the apparent molecular weight was around 220 kD. The radiolabelled protein was identified using a specific antibody to collagen type IV. The 180 kD protein was digested by collagenase with various radiolabelled split products. These data showed that cultured GEC produced collagen type IV as found previously (Foidart *et al.*, 1980; Striker *et al.*, 1984). With Il-1 purified from monocyte supernatants, or recombinant Il-1, there was increased synthesis of collagen type IV. The basal collagen production and the response to Il-1 varied from culture to culture with increases ranging from 5–20 times (examples for different donors are shown in Table 1).

Epithelial cell proliferation in response to purified Il-1 or to monocyte supernatants did not occur (results not shown), even though the latter were efficiently stimulating the collagen synthesis. A similar dissociation of the ability of Il-1 to induce

cell growth, from its capacity to stimulate collagen synthesis has been shown for skin fibroblasts. Depending on the experimental conditions, Il-1 induced collagen synthesis, but not cell proliferation (Kähäri *et al.*, 1987). Since Il-1 is not only produced by activated monocytes, but also by glomerular mesangial cells, e.g. in response to the terminal complement components (Lovett *et al.*, 1987), we suggest that Il-1 plays a role as an intrarenal mediator, which, by modulating the collagen synthesis, might participate in the development of sclerosis, leading thereby to a chronic inflammatory response.

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