Virchows Archiv (1994) 424:581–586

ORIGINAL ARTICLE

Masako Yanagisawa · Hirokazu Imai
Yukitaka Fukushima · Tadashi Yasuda · Akira B. Miura
Yasushi Nakamoto

Effects of tumour necrosis factor α and interleukin 1 β on the proliferation of cultured glomerular epithelial cells

Received: 13 September 1993 / Accepted: 21 March 1994

Abstract Rat glomerular epithelial cells were cultured with human monocyte supernatant or with recombinant cytokines. A primary glomerular culture and a glomerular epithelial cell culture were made; supernatant from monocyte cultures derived from healthy humans, and recombinant tumour necrosis factor α (TNF α) or recombinant interleukin 1 β (IL-1 β) were added. Cell proliferation rates were assayed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. In serum-free media, consistent proliferation of glomerular epithelial cells (GEC) was observed throughout the 3 week culture period. Significant growth-stimulatory effects were induced by lipopolysaccharide-treated monocyte conditioned medium and by 1–50 ng/ml of TNF α, growth being up to 400% more than in the control culture. The effect of TNF α depended mainly on its interaction with epidermal growth factor (EGF). In contrast to TNF α, IL-1 β inhibited GEC proliferation; this was due to the early appearance and proliferation of mesangial cells, despite the culture being serum-free. This study showed that activated monocytes secrete growth factors for GEC in vitro, and that interaction between both TNF α and IL-1 β and between TNF α and EGF can modulate GEC proliferation. These findings suggest that, under pathological conditions, monocytes or macrophages affect GEC proliferation, probably being involved in crescent formation.

Key words Glomerular epithelial cell culture
Monocyte · Tumour necrosis factor α · Interleukin 1 β
MTT assay

M. Yanagisawa (✉)
Arc Clinic of Cardiology and Nephrology,
26-1 Yachioki Hiroomote, Akita City,
Akita, 010, Japan

H. Imai · Y. Fukushima · T. Yasuda · A. Miura · Y. Nakamoto
Third department of Internal Medicine,
Akita University School of Medicine,
Akita, Japan

Introduction

Extensive cellular crescent formation in glomeruli has long been recognized as of great prognostic significance in glomerulonephritis. Monocytes and macrophages are considered to play an important role in the mechanisms underlying this crescent formation [4, 12, 13]. These cells are known to produce such factors as platelet-activating factor, thromboxane A_2, leukotrienes, reactive oxygen species, interleukin 1 (IL-1), and tumour necrosis factor (TNF) [18]. Under pathological conditions, these factors contribute to glomerular inflammation by causing glomerular capillary wall injury and by activating endothelial cells. In crescentic glomerulonephritis, glomerular epithelial cells (GEC) show dynamic proliferation; monocytes/macrophages are thought to have a salient effect on this proliferation, since they are present in cellular crescents as the cellular component. However, which factor actually stimulates the proliferation of GEC is not yet clear.

We succeeded in obtaining long-term GEC growth from a primary glomerular culture and a subculture of GEC in serum-free medium. We then examined the effects of a supernatant of lipopolysaccharide (LPS)-treated activated monocytes, together with TNF α or IL-1 β, on the proliferation of the cultured GEC. Possible factors that induce crescent formation are discussed.

Materials and methods

In order to culture rat glomerular epithelial cells 4–12-week-old male Wistar rats were killed by being deeply anaesthetized with ether, and the kidneys were amply perfused, via the abdominal aorta, with phosphate-buffered saline (PBS, pH 7.2) to remove blood cells. The glomeruli were isolated by a sieving technique, using stainless mesh, sizes 80 (pore size 177 μm), 150 (105 μm), and 250 (62 μm). The protocols for animal experimentation described in this paper were approved by the Animal Research Committee at Akita University School of Medicine; the "Guidelines for animal experimentation" of the University were totally adhered to in all animal experiments.

Isolated glomeruli were cultured on a collagen-coated culture plate (type 1 collagen, Sumitomo Bakelite, Japan) in RPMI1640 supplemented with 1% bovine serum albumin (BSA), insulin 5 μg/ml, transferrin 5 μg/ml, selenite 10^{-8} M, epidermal growth factor (EGF) 10 ng/ml, penicillin G 100 U/ml, and streptomycin 100 μg/ml. The culture dishes were incubated at 37° C in an atmosphere of 95% air and 5% carbon dioxide (CO_2). After 10–12 days of incubation, cells growing out were trypsinized for 10 min at 37° C, washed three times in PBS, and dispensed, at 5×10^5 cells/dish, into collagen-coated culture dishes. Thereafter, cells were routinely passaged at intervals of 7–10 days; the medium was replaced after 3 days.

Phase contrast microscopy was used to evaluate the characteristics of the cultured glomerular cells. For the immunofluorescent study, cells were plated onto collagen-coated coverslips and allowed to grow to the desired density. The coverslips were rinsed twice in PBS and fixed with cold acetone (–20° C) for 3 min and immediately air-dried. Rabbit IgG anti-cytokeratin (DAKO, USA), anti-human factor VIII (DAKO), and anti-rat Thy1-1 (Cedarlane Laboratories, Canada) antibodies were reacted with the coverslips for 30 min at 37° C. After the coverslips had been washed in PBS, they were reacted with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (DAKO) as the second antibody. The stained sections were examined under a universal fluorescent microscope (Zeiss, Oberkochen, Germany). For electron microscopy, cultures were fixed in 2% buffered glutaraldehyde and embedded in situ in epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and studied under an electron microscope (Nippon Electric 100B).

Mononuclear cells were separated from heparinized human peripheral blood on a Ficoll-Conray gradient, after which monocytes were enriched by fractionation on a one-step discontinuous Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient [1]. We confirmed that 92%–96% of the collected cells were monocytes by carrying out non-specific esterase staining. The monocytes (1×10^5/flask) were cultured at 37° C in humidified air with 5% CO_2 under RPMI1640 + 1% BSA+insulin 5 μg/ml + transferrin 5 μg/ml + selenite 10^{-8} M (medium A), or medium A plus LPS 10 μg/ml, as an activator for monocytes. The supernatants were collected after 5 or 48 h culture and stroed at –80° C until they were used.

The monocyte supernatant was added to the media of both the primary serum-free glomerular culture and the subcultured GEC, at concentrations of 5%, 10%, 20%, or 30%. Recombinant human IL-1 β (0.1–100 ng/ml; Otsuka Assay, Japan) or recombinant human TNF α (0.1–100 ng/ml; Genzyme, USA) were also added to both cultures. Five days after the addition of these factors, the number of cells was counted by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, USA) assay. The characteristics of the growing cells were analysed by phase contrast microscopy, electron microscopy, and immunofluorescent microscopy.

Cell proliferation was measured by MTT assay. Primary serum-free glomerular cultures or cells at passages 3–5 were used for this experiment. Forty glomeruli or 800 glomerular epithelial cells per well were cultured on 96-well collagen-coated tissue culture plates. On the fifth day of culture, 10 μl of MTT stock solution, prepared by dissolving 5 mg/ml of MTT in PBS and filtering the solution through a 0.22-μm filter (Whatmann, USA), was added to 100 μl of medium in the plates. The plates were then incubated at 37° C for 4 h. After the supernatant and glomeruli were removed, acid isopropanol (100 μl of 0.04N hydrochloric acid in isopropanol) was added to all wells, and the well contents were mixed thoroughly until the dark blue crystals dissolved. The plates were measured on an enzyme-linked immunosorbent assay reader (BioRad, Switzerland), using a measurement wavelength of 570 nm and a reference wavelength of 620 nm.

Data are given as means ± standard deviation for six separate experiments. Differences between groups were compared by a one-way analysis of Student's *t*-test. Differences were considered to be significant when $P<0.05$.

Results

Figure 1a shows polygonal cells in serum-free media on the 15th day of the primary glomerular culture; the cells formed a cobblestone-like monolayer. The proliferating cells were sensitive to puromycin aminonucleoside (Sigma), and, in the immunofluorescent studies, they revealed cytoplasmic cytokeratin (Fig. 2a), and negative staining for factor VIII and Thy1-1. Electron microscopic examination demonstrated microvilli and tight cell junctions, but there was no sign of microfilament bundles in the cells (Fig. 2b). Angiotensin-converting enzyme, a marker of endothelial cells, was not detected in the supernatant. These findings showed that the monolayer cells were GEC [27]. They proliferated consistently for 3 weeks; no mesangial cells proliferated. They had the same characteristics after they were subcultured, and we considered the purified cells also to be GEC (Fig. 1b).

The MTT assay reflects viable cell numbers [3, 16, 23, 30, 31]; the proliferation rates were expressed by optical density (*O.D.*) values for wave absorbance. Figure 3 shows the growth curve of glomerular epithelial cells at a cell count of 40–7000 per well. Cells were counted under a phase contrast microscope just before the MTT assay

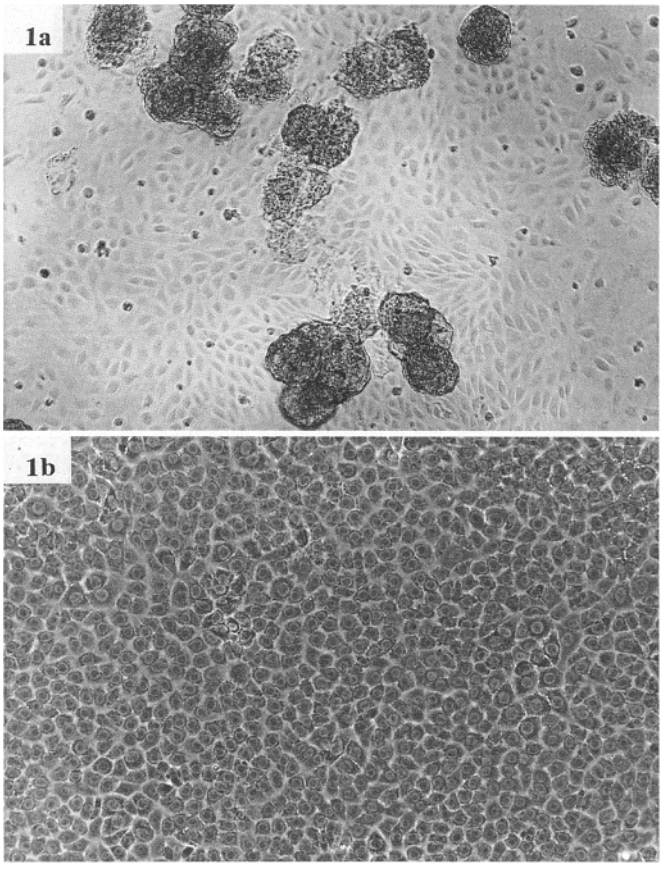

Fig. 1 **a** Primary serum-free glomerular culture on 15th day. Cobblestone-like polygonal cell proliferation is observed. Phase contrast microscopy (× 400). **b** Cells at the fifth passage on day 14 after subculture. Phase contrast microscopy (× 400)

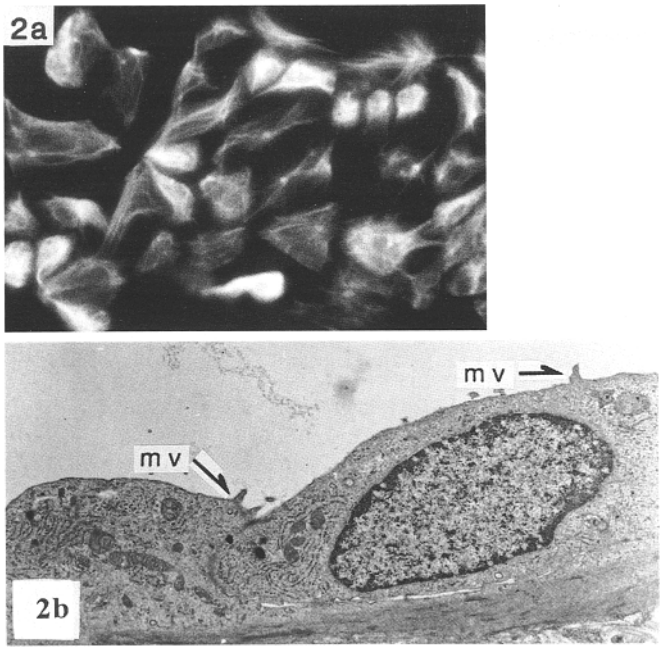

Fig. 2 a Positive immunofluorescent staining for cytokeratin (× 400). **b** Electron microscopy demonstrates microvilli (*mv, arrow*) and tight junction (× 11000)

was performed. There was an almost linear relationship between the cell count and the measured *O.D.*, the highest *O.D.* value being 0.310 ± 0.026.

For the assay of the primary glomerular culture, we removed glomeruli by vigorous pipetting before adding the acid isopropanol; MTT uptake by glomeruli was, thus, negligible, and we considered *O.D.* values to reflect the number of living GEC.

Phase contrast microscopy showed that the proliferation of monolayer cells in both the primary glomerular culture and the purified glomerular epithelial cells was greatly stimulated by the supernatant of LPS-stimulated monocytes. The results of the MTT assay were consistent with the microscopic findings. The mean *O.D.* value for the MTT assay of the control glomerular epithelial culture was 0.0315 ± 0.006. The proliferation rates of GEC were significantly increased with the supernatant of LPS-stimulated monocytes (Fig. 4). The highest proliferative rates of GEC were observed with the LPS-stimulated monocyte supernatant at a concentration of 20%, when the *O.D.* values were 0.132 ± 0.005 (48 h cultured monocytes; $P<0.005$ versus control) and 0.095 ± 0.016 (5 h cultured monocytes; $P<0.01$).

In the experiments with the two recombinant monokines, TNF α, at concentrations between 1 and 50 ng/ml, had stimulatory effects on the proliferation of GEC, in both the primary and the purified cultures. The *O.D.* value of the MTT assay in the control culture was 0.048 ± 0.019, and significant proliferation was brought about with 50 ng/ml of TNF α (*O.D.* value 0.109 ± 0.034, $P<0.01$). Although the effect of TNF α was reduced in culture medium without EGF, mild growth stimulation was still observed (Fig. 5).

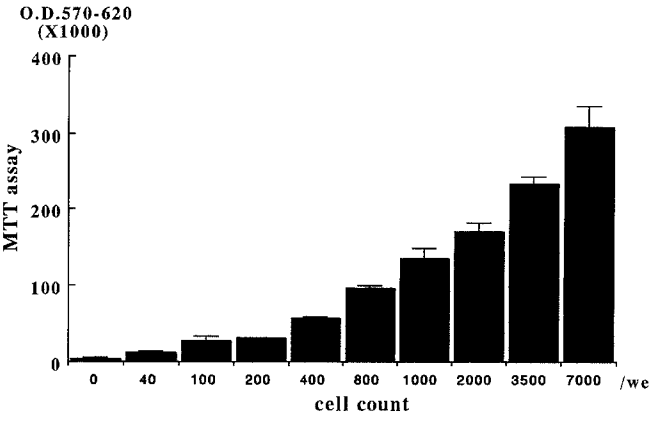

Fig. 3 MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay showing the growth curve of glomerular epithelial cells (GEC) at a cell count of 40–7000 per well

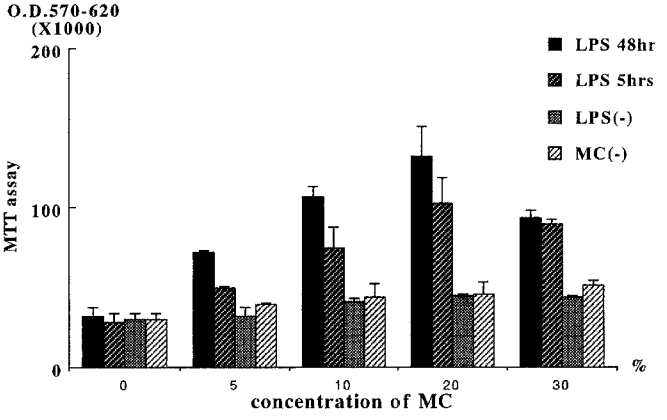

Fig. 4 MTT assay showing the effects of monocyte-conditioned medium on the proliferation of purified GEC. *MC* monocyte-conditioned medium, *MC*(−) growth of GEC without monocyte-conditioned medium, but with the same concentration of basal media as that used for the monocyte culture, *LPS*(−) growth of GEC with monocyte-conditioned medium not treated with lipopolysaccharide GEC proliferation was greatly stimulated by the addition of LPS-treated MC. Significant difference from control, $P<0.005$ (48 h treated MC); $P<0.01$ (5 h treated MC)

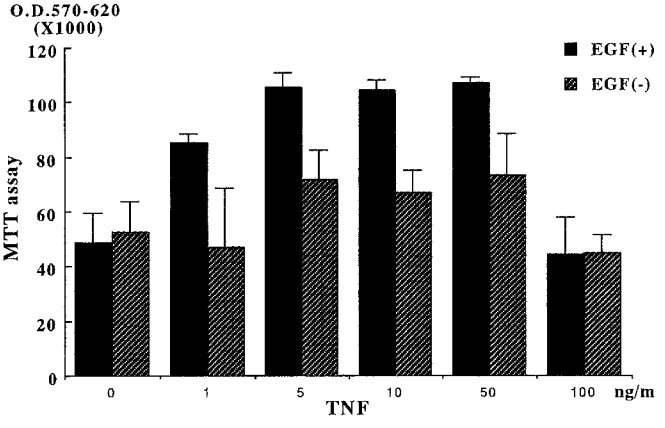

Fig. 5 MTT assay showing the effects of tumour necrosis factor α (*TNF* α) on the proliferation of cultured GEC. Proliferation rate was elevated with TNF α in the presence of epidermal growth factor (*EGF*). Significant difference from control, $P<0.01$

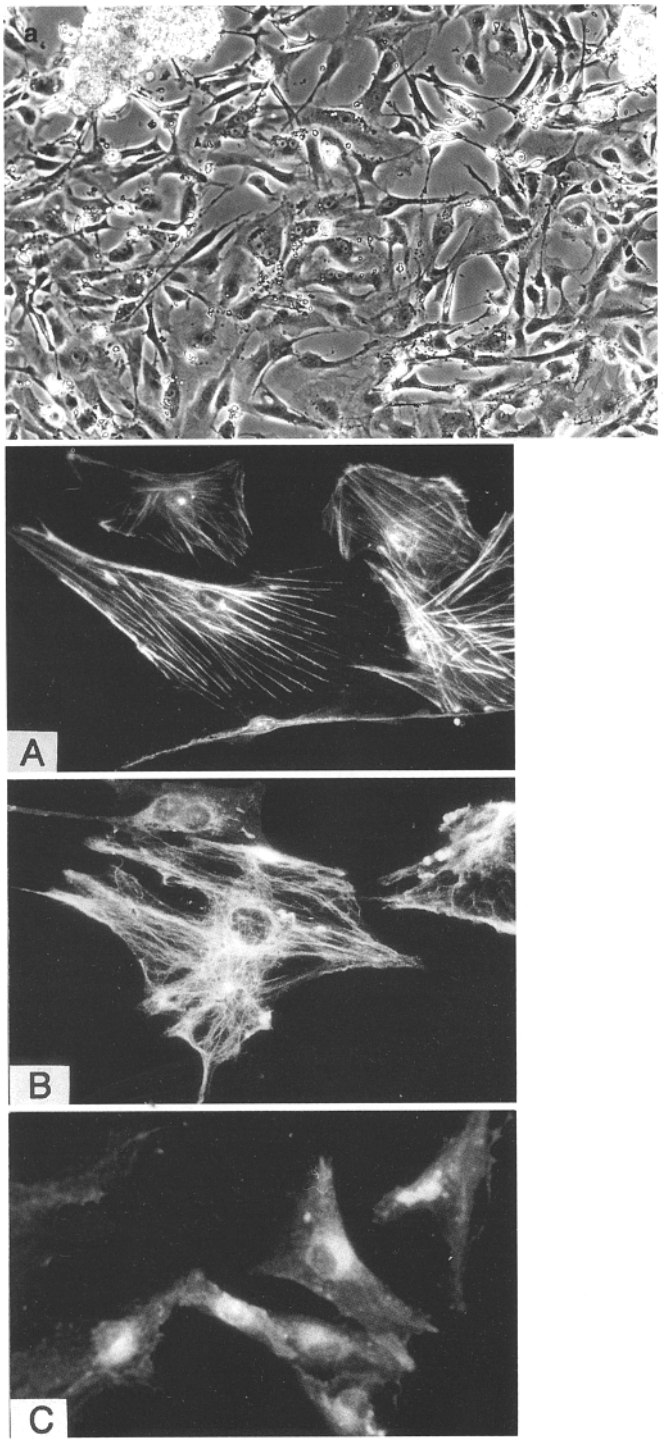

Fig. 6 **a** Primary glomerular culture with interleukin 1 (IL-1) β, day 5. GEC did not proliferate, and instead, mesangial cells appeared. **b** Immunofluorescent staining showing the dominant cells in primary glomerular culture treated with IL-1 β. **A** anti-actin, **B** anti-desmin, **C** anti-Thy1-1. (× 250)

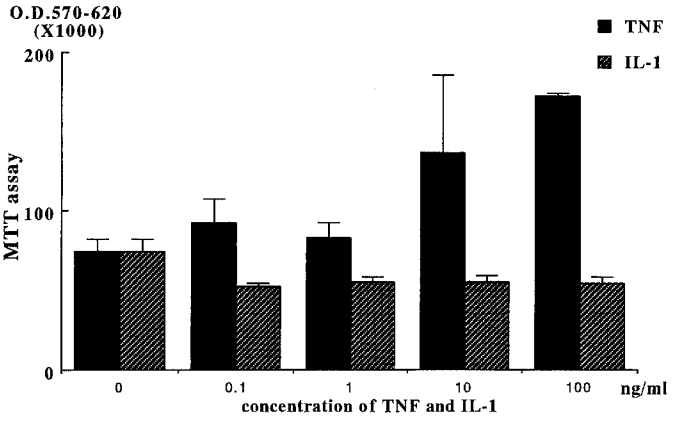

Fig. 7 Comparison of the effects of *TNF* α and *IL-1* β on the proliferation of purified GEC. IL-1 β had no effect. MTT assay

In contrast, no GEC grew in the media with added IL-1 β; instead, phase contrast microscopy showed that spindle-shaped cells appeared on the 3rd to the 4th day, despite the lack of fetal calf serum (FCS) in the culture medium (Fig. 6a). The immunofluorescent study demonstrated that these cells were positively stained with anti-actin, anti-desmin, and anti-Thy1-1 antibodies (Fig. 6b), but all were negative for cytokeratin and factor VIII. Electron microscopy revealed microfilament bundles in the cells. These findings indicate that, with the addition of IL-1 β, the growing cells were mesangial [33].With the purified GEC, TNF α again had a stimulatory effect on growth, while IL-1 β had no effect on their proliferation (Fig. 7).

Discussion

Kreisberg et al. [7] established techniques for culturing glomerular cells in 1978. They showed that when 20% FCS was used, GEC were the dominant growing cells during the early phase of culture, but that, after the 14th day, mesangial cells were predominant in the culture dish [6]. In 1984, Harper et al. [5] proposed an improved method for culturing GEC, using 5% FCS and collagen gel, since the EC grew best on a collagen gel-coated substratum with low serum (5%), while mesangial cells did best on plastic and in a high concentration of serum (20%) [5, 20]. It was then revealed that changes of the dominant cell type from glomerular epithelial to mesangial depended on the concentration of FCS. Many early investigators believed that cells growing during the early phase were visceral epithelial cells (VEC), since more than 85% of the glomeruli originally isolated were non-encapsulated [21]. However, Nørgaard [19] demonstrated three distinct cell types; parietal epithelial cells (PEC), VEC, and mesangial cells, in the primary glomerular culture, and concluded that PEC proliferated for approximately 2 weeks, and then reached a steady state, while VEC, as shown by light microscpic autoradiography, had only a limited proliferative capacity. It is therefore reasonable to consider that the proliferating GEC in our culture were PEC.

In active glomerulonephritis, proliferating PEC are observed in cellular crescents. Monocytes/macrophages are suspected of contributing to cellular crescent formation by inducing the proliferation of glomerular PEC and

fibroblasts [25]. Monocytes/macrophages, stimulated by LPS, produce growth factors for cultured mesangial cells [9, 10], IL-1 β and TNF α being the major reported factors; they are thought to play important roles in mesangial cell proliferation, as well as in the progression of glomerulonephritis [15, 24]. In this study, we demonstrated that LPS-treated monocytes secreted factors that were responsible for the proliferation of glomerular epithelial cells. Although we did not identify these factors, we consider that they are probably the cytokines IL-1 β and TNF α.

In further experiments that we carried out to confirm this possibility, we found that recombinant human TNF α, as expected, had a stimulatory effect on the growth of GEC. However, when recombinant IL-1 β was added to primary glomerular cultures with FCS-free medium, the dominantly growing EC were replaced by mesangial cells on the 6th day. The structures of TNF α and IL-1 β are very similar, and the two cytokines have multiple overlapping biological activities in cell activation and mitogenic action [2, 8]. Their stimulatory effects on collagen synthesis in GEC have also been reported [29]. The different action of TNF α and IL-1 β on GEC proliferation is interesting when one considers their similarities; it is possible that this difference is related to their activity in relation to either EGF or EGF receptors, as the proliferation rate of EC stimulated by TNF α was diminished by the omission of EGF from the culture medium. It has been reported that, under appropriate conditions, TNF α increases the responsiveness of cells to the mitogenic effect of EGF, and that this effct is correlated with the increased expression of cellular receptors for EGF in TNF-treated cells [22]. However, IL-1 has not been reported to interact with EGF. Therefore, it is reasonable to assume that the different actions of TNF α and IL-1 β onGEC are due to differences in their activity with either EGF or the EGF receptor. It has been reported that EGF alone had no direct mitogenic effects on glomerular cells [17], but that it had synergistic growth stimulatory effects with a certain type of cytokine [28]. Further, EGF or EGF-like peptides are derived from platelets and macrophages, and are produced by activated monocytes, playing an important role in the formation of cellular crescents.

Another interesting matter is the selective effect of IL-1 β on mesangial cell proliferation. Mazzei et al. [14], using neutralizing antibodies, reported the presence of 5–10 ng/ml of IL-1 in FCS, FCS being the most important growth factor for mesangial cells. IL-1 β also promotes cultured mesangial cell growth, and an autocrine IL-1-like substance obtained from growing mesangial cells has been investigated [11, 26]. Our findings suggest that IL-1 β has a selective stimulatory effect on the growth of mesangial cells, and that this selectivity is strong enough to result in the inhibition of GEC proliferation in the early phase in serum-free glomerular cultures.

We have shown that factors derived from monocytes activated by LPS acted as growth factors for GEC in vitro. When performing studies on GEC cultures, it must be realized that the origin of the EC is not certain, since the cells are derived from a mixture of whole glomeruli. However, our results support the hypothesis that, under inflammatory conditions, activated monocytes/macrophages infiltrate Bowman's space and then secrete factors that stimulate GEC proliferation, resulting in crescent formation. Further, we have shown that GEC proliferated in response to stimulation by the cytokines TNF α and IL-1 β. TNF α stimulates crescent formation, while IL-1 β inhibits it. It has also been shown that TNF α stimulates GEC to secrete several other potent mediators, such as a chemotactic factor for blood cells [32], which might be involved in the development of inflammatory glomerular lesions. Although the mechanisms underlying crescent formation are complicated, further exploration of the effects of the interaction between these factors on glomerular epithelial cells with throw some light on the pathophysiology and treatment of crescentic nephritis.

References

1. Colotta F, Peri G, Villa A, Mantovani A (1984) Rapid killing of actinomycin D-treated tumor cells by human mononuclear cells. I. Effectors belong to the monocyte-macrophage lineage. J Immunol 132(2):936–944
2. Freundlich B, Bomalaski JS, Neilson E, Jimenez SA (1986) Regulation of fibroblast proliferation and collagen synthesis by cytokines. Immunol Today 7:303–307
3. Gerlier D, Thomasset N (1986) Use of MTT colorimetric assay to measure cell activation. J Immunol Methods 94:57–63
4. Hancock WW, Atkins RC (1984) Cellular composition of crescents in human rapidly progressive glomerulonephritis identified using monoclonal antibodies. Am J Nephrol 4:177–181
5. Harper PA, Robinson JM, Hoover RL, Wright TC, Karnovsky MJ (1984) Improved methods for culturing rat glomerular cells. Kidney Int 26:875–880
6. Kreisberg JI, Karnovsky MJ (1983) Glomerular cells in culture. Kidney Int 23:439–447
7. Kreisberg JI, Hoover RL, Karnovsky MJ (1978) Isolation and characterization of rat glomerular epithelial cells in vitro. Kidney Int 14:21-30
8. Le J, Vilček J (1987) Biology of disease. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 56:234–248
9. Lovett DH, Sterzel RB (1986) Cell culture approaches to the analysis of glomerular inflammation. Kidney Int 30:246–254
10. Lovett DH, Ryan JL, Sterzel RB (1983) Stimulation of rat mesangial cell proliferation by macrophage interleukin 1. J Immunol 131:2830–2836
11. Lovett DH, Szamel M, Ryan JL, Sterzel RB, Gemsa D, Resch K (1986) Interleukin 1 and the glomerular mesangium. I. Purification and characterization of mesangial cell-derived autogrowth factor. J Immunol 136:3700–3705
12. Magil AB (1985) Histogenesis of glomerular crescents: immunohistochemical demonstration of cytokeratin in crescent cells. Am J Pathol 120:222–229
13. Magil AB, Wadsworth LD (1982) Monocyte involvement in glomerular crescent. A histochemical and ultrastructural study. Lab Invest 47:160–166
14. Mazzei GJ, Winger L, Osen-Sand A, Bernasconi L, Benotto W, Shaw AR (1989) Interleukin 1 alpha, a major component of fetal calf serum: artifacts in IL-1 bioassays. Cytokine 1:138
15. Monga G, Mazzucco G, Castello R (1985) Glomerular monocyte infiltration in human nephropathies: prevalence and correlation with clinical and morphological variables. Virchows Arch [A] 405:483–496

16. Mossmann T (1983) Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assay. J Immunol Methods 65:55–63
17. Nakagawa S, Yoshida S, Hirano Y, Kasuga S, Fuwa T (1985) Biological effects of biosynthetic human EGF on the growth of mammalian cells in vitro. Differentiation 29:284–288
18. Nathan CF (1987) Secretory products of macrophages. J Clin Invest 79:319–326
19. Nørgaard JOR (1987) Rat glomerular epithelial cells in culture. Parietal or visceral epithelial origin? Lab Invest 57:277–290
20. Oberly TD, Murphy-Ullrich JE, Steinert BW, Vic Muth J (1981) The growth of primary glomerular cells as a confluent monolayer in a chemically defined serum-free medium. Am J Pathol 104:181–188
21. Oberly TD, Steinert BW, Yang A-H, Anderson PJ (1986) Kidney glomerular explants in serum-free media. Sequential morphologic and quantitative analysis of cell outgrowths. Virchows Arch [B] 50:209–235
22. Palombella VJ, Yamashiro DJ, Maxfield FR, Decker SJ, Vilček J (1987) Tumor necrosis factor increases the number of epidermal growth factor receptors on human fibroblasts. J Biol Chem 262:1950–1954
23. Pieters R, Huismans DR, Leyva A, Veerman AJ (1989) Comparison of the rapid automated MTT-assay with a dye exclusion assay for chemosensitivity testing in childhood leukaemia. Br J Cancer 59:217–220
24. Radeke HH, Meier B, Topley N, Floge J, Habermehl GG, Resch K (1990) Interleukin 1-β and tumor necrosis factor-α induce oxygen radical production in mesangial cells. Kidney Int 37:767–775
25. Salant DJ (1987) Immunopathogenesis of crescentic glomerulonephritis and lung purpura (nephrology forum). Kidney Int 32:408–425
26. Sedor JR, Nakazato Y, Konieczkowski M (1992) Interleukin-1 and the mesangial cell. Kidney Int 41:595–599
27. Striker GE, Striker LJ (1985) Biology of disease: glomerular cell culture. Lab Invest 53:122–131
28. Takeuchi A, Yoshizawa N, Yamamoto M, Sawasaki Y, Oda T, Senoo A, Niwa H, Fuse Y (1992) Basic fibroblast growth factor promotes proliferation of rat glomerular visceral epithelial cells in vitro. Am J Pathol 141:107–116
29. Torbohm I, Berger B, Schönermark M, Kempis JV, Rother K, Hänsch GM (1989) Modulation of collagen synthesis in human glomerular epithelial cells by interleukin 1. Clin Exp Immunol 75:427–431
30. Twentyman PR, Luscombe M (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56:279–285
31. Visica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR (1991) Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Res 51:2215–2520
32. Watanabe K, Nakagawa H (1990) Cytokines enhance the production of a chemotactic factor for polymorphonuclear leukocytes by rat renal glomerular epithelioid cells. Nephron 54:169–175
33. Yaoita E, Kazama T, Kawasaki K, Miyazaki S, Yamamoto T, Kihara I (1985) In vitro characteristics of rat mesangial cells in comparison with aortic smooth muscle cells and dermal fibroblasts. Virchows Arch [B] 49:285–294