Immunology 1985 **56** 695

Modulation of mouse mesangial cell proliferation by macrophage products

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Accepted for publication 18 July 1985

Summary. Mesangial hypercellularity is usually found in many models of nephritis characterized by monocyte/macrophage infiltration of the glomerulus. In order to examine the mechanism mediating these events, an *in vitro* model was used to study the effects of macrophage products on mouse mesangial cells, cultured under conditions which would render them relatively quiescent. Under these conditions, macrophage supernatants stimulated the proliferation of the mesangial cells. The stimulatory effect could be shown to be due in part to enhancement of endogenous mesangial cell PGE production. This was demonstrated by experiments which showed that macrophage supernatants stimulated mesangial cell PGE production, that the stimulatory effect of macrophage products was abrogated by pretreatment of mesangial cells with indomethacin, and finally that exogenous PGE_2 stimulated mesangial cell proliferation.

INTRODUCTION

Recent studies have emphasized the role of the monocyte/macrophage as an effector cell in the pathogenesis of nephritis. Thus, in both experimental models of nephritis, namely anti-GBM nephritis and soluble immune complex mediated nephritis, monocytes have been found to be the predominant cells infiltrating the glomerulus (Schreiner *et al.*, 1978; Hunsicker *et al.*, 1979; Holdsworth, Neale & Wilson, 1981). Furthermore, if the influx of monocytes/macrophages is prevented by pretreatment of the experimental animal by irradiation or by the use of an anti-macrophage serum, the proteinuria seen in these models of nephritis is largely abrogated, suggesting that monocytes/macrophages produce glomerular injury by as yet unidentified mechanisms (Schreiner *et al.*, 1978; Hunsicker *et al.*, 1979; Holdsworth *et al.*, 1981). Another hallmark of many forms of nephritis is mesangial hypercellularity but the mechanisms responsible for this event are presently unknown.

In order to investigate the influences which monocytes/macrophages exert on mesangial cell proliferation, *in vitro* cell culture models were used to examine the effects of macrophage products on cultured mesangial cell growth (Ooi *et al.*, 1983a). Our initial studies showed that macrophage products exerted a suppressive effect on mesangial cell proliferation (Ooi *et al.*, 1983a). Because mesangial hypercellularity is more usually found in *in vivo* nephritis, we have re-examined the model using different conditions. In our initial studies, mesangial cells were grown in medium containing 20% fetal calf serum and, under these conditions, mesangial cells were in a relatively rapid state of division. To render the mesangial cells relatively more quiescent, the present studies were conducted culturing mesangial cells in medium containing 2·5% fetal calf serum; under such conditions, macrophage pro-

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ducts could be shown to exert a stimulatory effect on mesangial cell proliferation. It was also shown that enhancement of endogenous mesangial PGE production was one of the molecular mechanisms by which macrophage supernatants exerted this stimulatory effect. Thus, experiments were done which demonstrated that macrophage products stimulated mesangial cell PGE synthesis, that the stimulatory effect of macrophage products were abrogated by prior treatment of mesangial cells with indomethacin, and finally that exogenous PGE_2 stimulated mesangial cell proliferation.

MATERIALS AND METHODS

Cultivation of mouse mesangial cells

The procedures for cultivation and the characteristics of mouse mesangial cells have been previously reported in detail (Ooi *et al.*, 1983a). In brief, glomeruli were isolated from male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) 12–20 weeks old by a sieving technique (Barcelli *et al.*, 1981). Glomeruli were plated onto 30 ml tissue culture flasks (Corning Labware, Corning Glass Works, Corning, NYH) containing Dulbecco's Modified Eagle (DMEM, Grand Island Biological, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) in 37° humidified 5% CO_2 incubator. This medium will be designated complete DMEM. Cellular outgrowths were observed 10–14 days after seeding. Cultures were fed biweekly and transferred at confluence.

The characteristics of the mouse mesangial cells have been described previously in detail (Ooi *et al.*, 1983a). Cells were used when they were apparently homogenous as assessed by phase contrast and by light microscopy and by their staining characteristics. Thus, >90% of the cells reacted by indirect immunofluorescence with anti-mouse fibronectin (gift from Dr E. Engvall, La Jolla Cancer Research Foundation, La Jolla, CA) and with monoclonal antibody to smooth muscle actin (gift from Dr J. Lessard, Cincinnati Children's Hospital Research Foundation). The latter antibody was prepared against chicken gizzard and was not reactive with fibroblasts. Contamination with monocytes and with lymphocytes was excluded by absence of reactivity with anti-Mac 1 (gift from Dr T. Springer, Harvard Medical School and Hybritech, San Diego, CA) anti-Thy 1,2 (Litton Bionetics, Kensington, MD) and monoclonal anti-Ia (supernatant of clone M5/114.15.2 American Type Culture Collection, Rockville, MD) as assessed by indirect immunofluorescence. The cells had the ultrastructural characteristics of mesangial cells reported by Ausiello *et al.* (1980). They had oval nuclei, abundant microfilaments parallel to the long axis of the plasma membrane and exhibited surface dense patches. The cells were not sensitive to the addition of the aminonucleoside of puromycin and their growth characteristics were unchanged in D-valine substituted medium, indicating further that they were not fibroblasts (Gilbert & Mignon, 1975). By cell labelling techniques, they could be shown to synthesize fibronectin (Ooi, Weiss & Ooi, 1983b).

Macrophage cultures and preparation of supernatant

Male C57BL/6J mice, 12–20 weeks old were killed by cervical dislocation and were injected i.p. with 10 ml of calcium and magnesium-free Dulbecco's phosphate-buffered saline (PBS). The resulting cell suspensions were pooled and were centrifuged at 800 rpm for 10 min at room temperature; the cells were then resuspended in complete DMEM. Washed peritoneal cells ($1{\cdot}4 \times 10^7$ per dish) were dispensed onto plastic petri-dishes (60 × 10 mm, Falcon Plastics Division, Oxnard, CA) and incubated at 37° in 5% CO_2 humidified atmosphere for 1 hr; non-adherent cells were then removed by four successive washes with complete DMEM. In separate cultures, adherent cells were defined as more than 90% macrophages as monitored by ingestion of latex particles and sheep erythrocytes coated with antibody. Adherent cells were fed with 2 ml of complete DMEM containing 2·5% fetal calf serum and were incubated for another 24 hr, after which the supernatant was aspirated and centrifuged at 400 ***g*** for 10 min, and the cell-free supernatant obtained was dialysed against media and stored at −70° until use.

Assay for [^{3}H]thymidine uptake by mesangial cells

Confluent mesangial cell cultures were harvested in the following manner. The medium was aspirated and the cells were washed twice with Hanks' balanced salt solution (HBSS, Grand Island Biological, Grand Island, NY) and treated with 0·6 ml of 0·5% trypsin-0·5 mM EDTA solution (Grand Island Biological, Grand Island, NY) for 15 min at 37°C. Mesangial cells were then resuspended in complete DMEM containing 2·5% fetal calf serum and were adjusted to a concentration of 1×10^5 cells/ml. A concentration of

2·5% FCS was chosen arbitrarily as one in which cells would grow, but at a markedly reduced rate compared to its growth at 20% FCS, a concentration used by us in previous experiments (Ooi *et al.*, 1983a). An aliquot of 1×10^4 mesangial cells was added to varying volumes of macrophage supernatant or complete DMEM in wells of flatbottomed tissue culture plates (Microtest II, Falcon Labware) and was cultured for 48 hr in a 5% CO_2 humidified atmosphere at 37°. An aliquot of [^{3}H]-thymidine (20 Ci/mmol, New England Nuclear, Boston, MA) was added to each culture for the final 16 hr. At termination of the culture, medium was aspirated and cells were washed twice with HBSS. An aliquot of 50 μl of 0·05% trypsin with 0·5 mM EDTA (Grand Island Biological, Grand Island, NY) was added to each well; cells were harvested onto glass fibre filters and were washed with distilled water with a semi-automatic cell harvester (Titertek Cell Harvester, Flow Labs, Inc., Rockville, MD). Filter discs were punched out, transferred to scintillation vials containing Aquasol-2 (New England Nuclear, Boston, MA) and counted in a LS-7000 Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA). All experiments were done in triplicate or more.. For experiments assessing the effect of indomethacin, experiments were performed in an identical fashion except that mesangial cells were pretreated with indomethacin (10 μg/ml) in alcohol (final concentration 0·1%) for 5 hr before adding supernatant or control medium.

Assay for effect of macrophage supernatant on PGE synthesis

For these experiments, 0·3 ml of macrophage supernatant or control medium was added to 1×10^5 cells in 0·9 ml of complete DMEM (2·5% FCS) and incubated for 48 hr. The supernatant was harvested and assayed for immunoreactive PGE by a radioimmunoassay according to the manufacturer's instructions Serapak, Lasagen Inc., Boston, MA). Statistical analysis was done by Student's *t*-test.

RESULTS

The effect of adding macrophage supernatant on [^{3}H]-thymidine uptake by mesangial cells cultured in 2·5% fetal calf serum is shown in Fig. 1. A dose-dependent stimulatory effect was observed. Statistical analysis showed that the mean [^{3}H]thymidine uptake of mesangial cells incubated with 2·5% concentration macro-

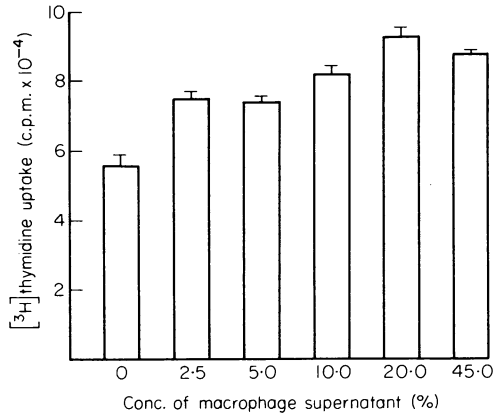

Figure 1. Effect of macrophage supernatants on mouse mesangial cell [^{3}H]thymidine uptake. The results show the means ± SE of quadruplicate determinations. Statistical analysis showed that the mean [^{3}H]thymidine uptake of mesangial cells grown in 2·5% macrophage supernatant or greater was significantly higher ($P < 0·01$) than that of cells grown in DMEM. Intramural comparisons also showed mean mesangial [^{3}H]thymidine uptake of cells grown at 20% and 45% macrophage supernatant to be significantly greater than that of cells grown in 5% macrophage supernatant or less.

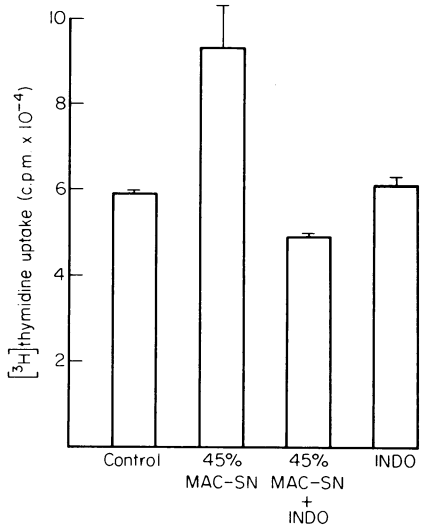

Figure 2. Effect of macrophage supernatant on mesangial cells pretreated with indomethacin. The mesangial cells were grown in 2·5% FCS and the results represent means ± SE of triplicate determinations. Note that pretreatment of mesangial cells with 5 μg/ml indomethacin abrogated the stimulatory effect of 45% macrophage supernatant (differences between the means of the two groups were statistically significant $P < 0·02$). Indomethacin in alcohol had no effect on mesangial cell growth.

phage supernatant or greater was significantly higher than that of control cultures ($P<0.01$). Intramural comparisons showed that mean [^{3}H]thymidine uptake of mesangial cells cultured with 20% and with 45% macrophage supernatant to be statistically greater than that of cells cultured with 5% macrophage supernatant or less ($P<0.01$).

The effect of preincubation of mesangial cells with indomethacin before the addition of macrophage supernatant is shown in Fig. 2. The addition of 45% macrophage supernatant to mesangial cell cultures caused a significant increase ($P<0.05$) in [^{3}H]thymidine uptake by the cells. Pretreatment of the cells with indomethacin abrogated this increase, the differences between the mean [^{3}H]thymidine uptake of cell cultured with 45% macrophage supernatant and those cultured with 45% macrophage supernatant and indomethacin being statistically significant ($P<0.02$). A control containing indomethacin in alcohol had no effect on cell growth.

To determine if macrophage supernatant stimulated PGE synthesis, supernatant of mesangial cells cultured in either control DMEM or macrophage supernatant was assayed for immunoreactive PGE by radioimmunoassay. The results (Table 1) showed that macrophage supernatant stimulated PGE production by mesangial cells.

Finally, the effect of exogenous PGE_2 on the growth of mesangial cells cultured with 2·5% FCS was determined. The results (Fig. 3) showed a stimulatory effect of PGE_2 on mesangial cell growth in a dose dependent fashion. Statistical analysis showed that the mean [^{3}H]thymidine uptake of mesangial cells cultured with all concentrations of PGE_2 was significantly greater than that of control values ($P<0.01$). Differences between means of successive experimental groups were also statistically significant ($P<0.05$) except for the following comparisons: PGE_2 1 μg/ml *vs* 2 μg/ml, and PGE_2 2μg/ml *vs* 4 μg/ml.

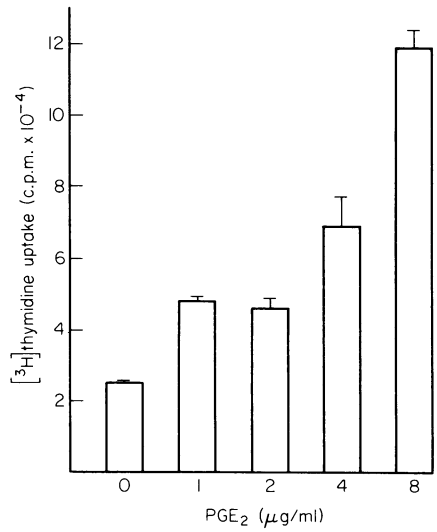

Figure 3. Effect of exogenous PGE_2 on mesangial cell growth. Cells were grown in 2·5% FCS and results represent means ± SE of quadruplicate determinations. Mean [^{3}H]thymidine uptake of mesangial cells cultured with PGE_2 was significantly higher compared to control uptake ($P<0.01$). Differences between means of successive experimental groups were also statistically significant ($P<0.05$) or greater, except for the following comparisons: PGE_2 1 μg/ml *vs* 2 μg/ml and PGE_2 2 μg/ml *vs* 4 μg/ml.

DISCUSSION

The current studies demonstrate that macrophage products stimulated the proliferation of mouse mesangial cells cultured in 2·5% fetal calf serum so that they were relatively more quiescent (compared to cells cultured in 20% fetal calf serum). The findings confirm earlier studies, reported in abstract form, showing such an effect of macrophage products on cultured mesangial cells (Nagle *et al.*, 1981; Werny *et al*, 1981). The present study also shows that one of the molecular

Table 1. Effect of macrophage supernatant on mesangial cell PGE production

	PGE (ng/nl)		
	Exp. 1	Exp. 2	Mean (PGE ng/ml)
Mesangial cells	3·0	2·7	2·9
Mesangial cell + 50% macrophage − SN	6·3	5·9	6·1
50% macrophage − SN	<0·5	<0·5	<0·5
50% DMEM	<0·5	<0·5	<0·5

mechanisms by which this effect is obtained is by stimulating endogenous mesangial cell PGE synthesis. Thus, experiments showed that macrophage supernatants stimulated mesangial cell PGE production, that the stimulatory effect of the supernatant could be abrogated by pretreatment of the target cells with indomethacin, and finally that exogenous PGE exerted a stimulatory effect on mesangial cell growth.

One of the substances produced by macrophages which has been shown to stimulate rat mesangial cell, human endothelial cell and human fibroblast proliferation (Nagle *et al.*, 1981; Lovett *et al.*, 1983a; Schmidt *et al.*, 1982; Ooi *et al.*, 1983a) is interleukin-1. The recent report that rat mesangial cells produce an interleukin-1-like substance (Lovett, Ryan & Sterzel, 1983b) indicates that there may be an amplification loop which occurs in monocyte/macrophage-mesangial cell interactions. Thus, if mesangial cells, stimulated to proliferate by monocyte interleukin-1 (and by other monocyte/macrophage products) produced even greater amounts of mesangial interleukin-1, proliferation of mesangial cells would be enhanced by such a mechanism. Clearly, negative feedback mechanisms must be summoned under these circumstances to regulate the proliferative stimuli released.

It is in this context that our earlier observations (Ooi *et al.*, 1983a) showing a suppressive effect of macrophage supernatants on cultured mouse mesangial cells, may have importance. When macrophage supernatants were added to mouse mesangial cells grown in 20% fetal calf serum such that they were rapidly dividing, the growth of these cells was suppressed. It is of interest that this suppressive effect was mediated in part by a mechanism which appeared to exert its effect by stimulating endogenous prostaglandin E synthesis. It would appear that the action of PGE on cell growth is dependent on the physiological state of the cell. Previous studies of the effects of macrophage products (Lovett *et al.*, 1983a; Korn, Haluska & LaRoy, 1980) on fibroblast growth support the present observations.

The present model lends itself to further studies of the effects of macrophage products on other parameters of mesangial cell physiology. It would be of importance to determine if many of the physiological derangements are caused by a single substance such as interleukin-1, or by a whole range of products. The precise identification of these molecular mechanisms will allow eventual therapeutic strategies to be devised to enable us to treat the various forms of nephritis.

ACKNOWLEDGMENTS

We are grateful to Doctors Eva Engvall, La Jolla Cancer Research Foundation, J. Lessard, Cincinnati Children's Hospital Research Foundation and T. Springer, Harvard Medical School, for generous gifts of antisera. The excellent secretarial assistance of Mary Grannen and Violet Henry is acknowledged.

This study was supported, in part, by a National Institutes of Health grant AM 34628.

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