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IL-1-like production in adriamycin-induced nephrotic syndrome in the rat

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SUMMARY

Rats receiving a single dose of adriamycin (7·5 mg/kg) develop heavy proteinuria and morphological abnormalities similar to those observed in minimal change nephrotic syndrome in humans. A concomitance between enhanced I-a display by resident glomerular macrophages, IL-1-like cytokine secreted by whole isolated rat glomeruli and proteinuria was observed in adriamycin-injected rats during the experimental protocol. In addition, *in vitro* studies have shown that after stimulation with adriamycin or lipopolysaccharide (LPS) this cytokine is mainly produced by resident glomerular macrophages in culture. Although the precise mechanism of proteinuria in this model needs to be further studied, our results indicate that IL-1-like cytokine could be an important mediator implicated in the structural and functional disturbances occurring at the glomerular capillary wall level in adriamycin nephrosis.

Keywords IL-1-like cytokine experimental nephrosis glomerular cell cultures mesangial cells resident glomerular macrophages I-a expression

INTRODUCTION

Rats receiving a single dose of adriamycin develop proteinuria and glomerular morphological abnormalities similar to those observed in minimal change nephrotic syndrome in humans [1–2]. In spite of the extensive studies done, the precise mechanism of proteinuria, at the cellular level, remains unresolved [3–4].

Special attention has been recently focused on glomerular mesangium, strategically situated among the capillary loops, as an active secretory structure releasing vasoregulatory soluble mediator factors [5–6]. Of particular interest is the demonstration that rat mesangial cells in culture [7] and lipopolysaccharide (LPS)-stimulated glomeruli [8] produce a factor which closely resembles macrophage IL-1.

In the present study we have found that whole glomeruli from rats with adriamycin-induced nephrosis release an IL-1-like cytokine. Further *in vitro* studies have also demonstrated that this cytokine is mainly produced by glomerular macrophage cells. The possible contributory role of IL-1 in the development of proteinuria is discussed.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats, 10 weeks old and weighing 180 g, were obtained from our own breeding colony, maintained in standard conditions and used throughout the experiment.

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Induction of experimental nephrosis

A group of 30 rats were injected in the tail vein with a single dose (7·5 mg/kg body weight) of adriamycin (Adriablastine, Farmitalia, Carlo Erba, Milan) dissolved in normal saline. Another group of 20 rats, injected only with saline, were used as controls. Animals were killed on days 7, 14, 21 and 28 after injections. Urine was collected from rats housed 24 h in metabolic cages with access to water only and the amount of proteinuria quantified by means of the Lowry method [9].

Kidney tissue processing

Animals were anaesthetized with 5 mg/100 g of sodium pentobarbital and perfused *in vivo*, under a laminar flow hood, as has been previously reported [10].

A piece of renal tissue was taken for light and electron microscopy studies. For light microscopy, kidney sections were fixed in 10% formalin and stained with haematoxylin and eosin (H & E). Tissue for ultrastructural examination was fixed in 4% glutaraldehyde, followed by post-fixation in 1% osmium tetroxide and further processed as described [11]. A part of renal cortex from each animal was snap-frozen in isopentane, precooled in liquid nitrogen. Glomerular cells were further characterized in frozen tissue sections by the avidin-biotin-peroxidase complex (ABC) technique, using MoAbs to recognize monocytes-macrophages (ED1) and I-a-bearing cells (OX6) (Serotec, Oxford, UK) as previously described [12,13]. The specificity of these monoclonal reagents was assessed using normal mouse serum, normal mouse IgG and hybridoma-induced ascitic fluids containing unrelated antibodies [14].

Using conventional light microscopy, the average number of positive cells per glomerulus for each animal was determined. An average total of 120 (range 80–145) glomeruli were counted for each MoAb in each animal.

Isolation and culture of glomeruli

Slices of renal cortex were pressed through graded sieves of decreasing pore size as previously described [15]. Preparations, consisting of >95% glomeruli, were suspended at a final concentration of 1×10^3 glomeruli/ml in serum-free medium (GIBCO, Grand Island, NY) and then incubated for 24 h in petri dishes (Corning Glass Work, Corning, NY) at 37°C in a 5% CO_2 atmosphere. At the end of the culture period, supernatants were harvested, centrifuged at 100 *g* and stored at −20°C until assayed for the release of IL-1-like cytokine.

Cell preparation and cultures

Normal control rat glomeruli, obtained as described above, were further processed for mesangial and resident macrophage cell cultures. For mesangial cell cultures, the glomeruli were incubated with collagenase Type IV (500 U/ml, Sigma Chemical Co, St Louis, MO) and DNAase Type III (10 μg/ml, Sigma) for 30 min at 37°C and suspended in culture medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% glutamine, 0·66 U/ml insulin, 1% HEPES and 1% antibiotics). Glomeruli (3×10^3/ml) were planted on to uncoated tissue culture flasks (Corning). When cell outgrowth reached confluence, mesangial cells were removed (0·025% trypsin-0·5 mM EDTA in calcium and magnesium-free Hank's balanced salt solution (HBSS)). Disassociated cells were resuspended in culture medium and placed again on to new tissue flasks. After three to four passages, supernatants were collected and stored at −20°C. Mesangial cells were identified by their characteristic morphological features, the presence of smooth muscle cell myosin and the absence of positivity for Factor VIII antigen (reagents obtained from Dakopatts, Copenhagen, Denmark), as revealed by the ABC technique. Less than 2% of these cells were contaminants. Before stimulation studies, washed monolayers of growing mesangial cells were incubated for 24 h in fresh serum-free medium.

Resident glomerular macrophages were obtained from glomeruli placed in a solution of collagenase Type IV (600 U/ml) for 60 min at 37°C in a rocking platform. The glomeruli were washed and subsequently placed in a solution of 0·1% trypsin-EDTA (GIBCO) for 30 min at 37°C. The disassociated cells were resuspended in DMEM (GIBCO) with 20% FCS at a concentration of 5×10^4 cells/ml and placed on glass cover-slips into 24-well tissue culture plates (Costar, Cambridge, MA) for 2 h at 37°C in 5% CO_2. Then, 1 ml of K1-3T3 Swiss medium, consisting of equal parts of K1 medium with 5% Nu Serum (Collaborative Research, Waltham, MA) and 24 h conditioned DMEM with 20% FCS from Swiss 3T3 fibroblasts [16], supplemented with 1% antibiotics, was added to each well. Before stimulation studies, washed macrophage cell monolayers were incubated for 24 h in fresh RPMI serum-free medium. Macrophage cells were identified by immunofluorescence staining with ED1 specific MoAb (Serotec) followed by FITC-F $(ab')_2$ goat anti-mouse IgG (Cappel, Cochranville, PA) and by non-specific esterase staining [17]. In addition, these cells were phagocytic when incubated for 1 h with *Candida albicans* [18]. The macrophage preparations were approximately 90% pure.

In vitro *stimulation studies*

Whole rat glomeruli, mesangial cells and resident glomerular macrophages were adjusted to 512 μg of protein content/well for each sample, using the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL). Then, samples were incubated in serum-free medium in the presence or absence of LPS (0·1–100 μg/ml) or adriamycin (10^{-5}–10^{-15} M). Supernatant fluids were collected and stored at −20°C.

Thymocyte proliferative assay

The proliferative response of lectin-primed mouse thymocytes was used to quantify IL-1 activity present within the different samples. Thymocytes were prepared from ether anaesthetized Balb/c mice and incubated in 60 mm plastic dishes (Corning) for 2 h at 37°C in 5% CO_2. Non-adherent cells were resuspended in RPMI medium and placed at a density of 1×10^6 cells/well on to 96-well tissue culture plates (Costar) in the presence or absence of 25 μg/ml of phytohaemagglutinin (PHA) (Wellcome Laboratory, Beckenham, UK). Serial dilutions of supernatants were added at a final volume of 200 μl/well. After 48 h of culture, the wells were pulsed with 1 μCi [^{3}H]-TdR and harvested 24 h later. Results were expressed as the mean ± s.d. of triplicated wells compare with controls containing mitogen alone. Recombinant human IL-1-beta (Genzyme Corp., Boston, MA) was used as IL-1 standard. Purified materials did not contain detectable IL-2 activity, as determined on CTLL-2 cells [7]. All test preparations were screened for the presence of endotoxin by using the limulus amoebocyte lysate (LAL) assay (Whittaker/M. A. Bioproducts, Walkersville, MD).

Characterization by molecular weight of IL-1-activity-associated molecules

Supernatants (3 ml) from each sample were applied to a 1·6 × 60 cm Sephacryl 200-SF column (Pharmacia, Uppsala, Sweden) previously equilibrated with apyrogenic Tris-buffer pH 7·4, and calibrated with the following standards: bovine serum albumin (BSA) (mol. wt 67·000), chymotrypsin (mol. wt 25·000), cytochrome c (mol. wt 12·400), and insulin (mol. wt 5·700). The column was eluted at a flow rate of 8 ml/h. Fractions of 0·5 ml were collected and assayed at 1/8 final dilution for the ability to enhance thymocyte proliferation.

Neutralization test

Rabbit anti-IL-1-beta (Genzyme) was pre-incubated at 5% concentration (v/v) with renal glomeruli supernatants and with purified recombinant human IL-1-beta (Genzyme) overnight at 4°C. The immunocomplexes formed were removed with Protein-G Sepharose (Pharmacia) and centrifuged at 11 000 *g* for 15 min. Next, the supernatants were applied to a 1·6 × 60 cm Sephacryl 200-SF column (Pharmacia). Fractions were collected and assayed for IL-1 activity.

Statistical analysis

Statistical differences among groups were assessed by using the two-tailed Student's *t*-test.

RESULTS

Proteinuria

Table 1 shows the time course of urinary protein excretion in adriamycin-treated rats and control rats. Mean protein excre-

Table 1. Protein excretion in adriamycin-induced nephrosis

Days post-injection	Proteinuria (mg/24 h)
7	38·75±10
14	241·21±35
21	477·03±55
28	380·12±24
Controls	6·25±03

Each value represents the arithmetic mean ±s.d. of protein excretion over 24 h of groups ranging between five and eight animals.

Table 2. Antigenic changes in adriamycin-induced nephrosis

Days post-injection	No. of positive cells/glomerulus		
	ED1	I-a	I-a/ED1
7	2·28±0·35	1·60±0·23	0·67
14	2·26±0·50	2·18±0·51	0·82
21	2·10±0·32	1·77±0·47	0·76
28	1·73±0·51	1·67±0·76	0·70
Controls	2·00±0·35	0·91±0·11	0·40

Mean ±s.d. of groups counted in 50 glomeruli of groups ranging between five and eight animals. All animals injected with adriamycin presented a significant increase in the number of I-a cells ($P<0·001$).

tion was 38·75±10 mg/24 h on day 7 and progressively increased to 477±55 on day 21 of the experiment. Thereafter, although a discrete decrease was noted, rats remained heavily proteinuric.

Histology and immunohistochemistry

Serial studies by light microscopy of glomeruli from rats treated with adriamycin, throughout the 4 weeks of this study, showed there were no apparent glomerular histological abnormalities. Ultrastructural examination demonstrated important alterations on glomerular epithelial cells with fusion of foot processes and detachment of epithelial cells from the underlying basement membrane.

Table 2 indicates the number of ED1$^+$ mesangial cells (macrophage cells) and the expression of I-a antigens in rat glomeruli from kidney frozen sections. There were no significant differences in our serial studies between the number of ED1$^+$ mesangial cells from rats injected with adriamycin and the controls. However, a significantly ($P<0·001$) enhanced display of I-a antigens by resident glomerular macrophages was observed in adriamycin rats, as is also illustrated in Table 2. An increased number of resident macrophages bearing I-a molecules was observed during the second and third week of disease, decreasing thereafter although continuing to remain higher than controls.

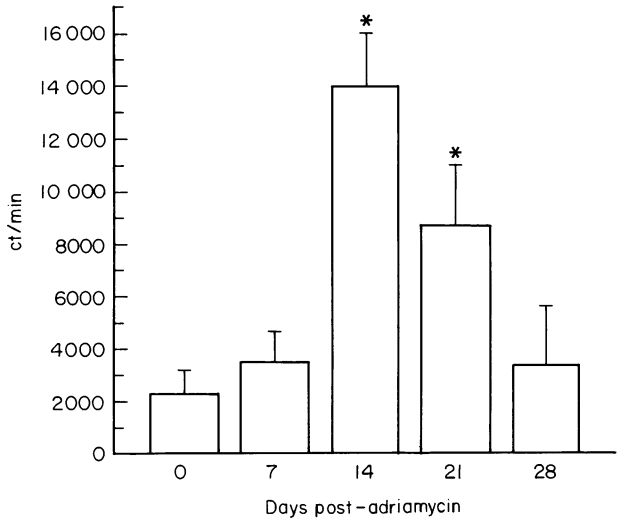

Fig. 1. IL-1-like activity released by glomeruli from adriamycin-induced nephrosis in the rat. *$P<0·001$, significantly greater than control rats.

Kinetics of IL-1-like cytokine production by isolated whole glomerular culture supernatants from adriamycin rats

As seen in Fig. 1, the mean levels of IL-1-like activity in the groups of rats treated with adriamycin were found to be significantly higher ($P<0·001$) during the experiment than those in controls. Maximal IL-1-like activity was detected on day 14 of the disease when enhanced I-a display by mesangial macrophages was highest and rats were heavily proteinuric.

The in vitro *effect of adriamycin on glomerular and cell cultures*

Stimulation of cultures with appropriate concentrations (10^{-9} M) of adriamycin, as determined by prior optimization experiments (see Fig. 2), results in the production of IL-1-like activity by all samples tested (Fig. 3). The mean levels of this activity from whole glomeruli and glomerular macrophage supernatant fluids were found to be significantly ($P<0·01$) higher than those from mesangial cells.

In this system, IL-1-like activity from LPS-activated resident glomerular macrophages was also found to be higher ($P<0·1$) than that produced by LPS-stimulated mesangial cells. In order to show that the activity produced by the glomerular cells was not due to carry-over of LPS, the direct effect of LPS on the proliferative assay was tested (Table 3). Positive control in the thymocyte proliferative assay was 13·432±321 ct/min for recombinant human IL-1-beta (1 U/ml) (Genzyme).

From our experimental results we have been able to demonstrate that glomerular IL-1-cytokine is mainly secreted by activated resident glomerular macrophages.

Characterization of IL-1-like cytokine

Concentrated supernatants from each sample were applied to a calibrated Sephacryl S 200-SF column and collected fractions tested in the thymocyte proliferation assay at 1/8 dilution. The activities from all samples were eluted in two areas corresponding to 10–20 kD and 5–8 kD. When the same samples were preincubated with rabbit anti-IL-1-beta antiserum, the biological activity of both areas showed a decrease of 80% (Fig. 4).

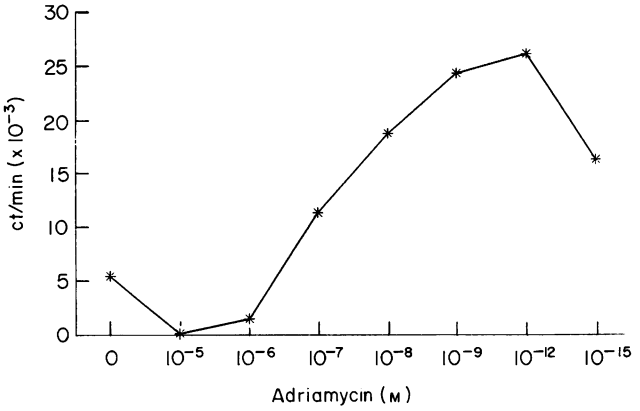

Fig. 2. Effect of graded doses of adriamycin on IL-1-like secretion from glomerular culture supernatants.

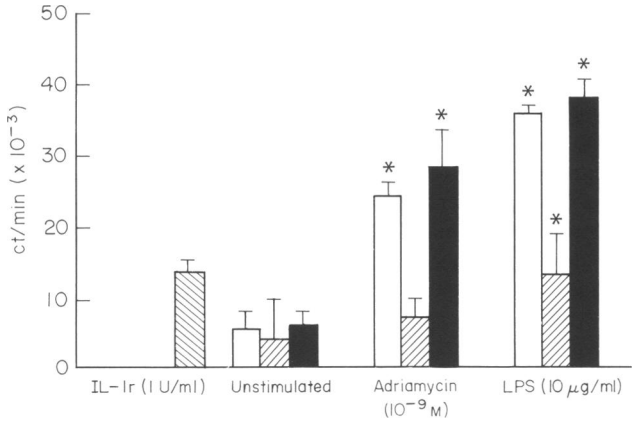

Fig. 3. Production of IL-1 activity in supernatants from whole isolated rat glomeruli and glomerular cells after *in vitro* stimulation. $*P < 0.01$. ▧, positive control; □, glomeruli; ▨, mesangial cells; ■, glomerular macrophages. LPS, lipopolysaccharide.

Table 3. Effect of different doses of lipopolysaccharide on thymocyte proliferative assay

LPS (μg/ml)	0	1	5	10	100
ct/min*	242±101	353±54	786±224	814±159	1162±286

* Each value represents the mean±s.d. of the direct effect of lipopolysaccharide (LPS) on proliferative assay.

DISCUSSION

The adriamycin model of nephrotic syndrome in the rat is well documented [1], although the precise mechanism leading to glomerular damage is still poorly understood [3–4]. Glomerular morphological abnormalities, as shown by ultrastructural studies, consist of loss of the normal foot process architecture and replacement by flattened epithelial cytoplasm [1]. Our studies have confirmed these findings and have also attempted

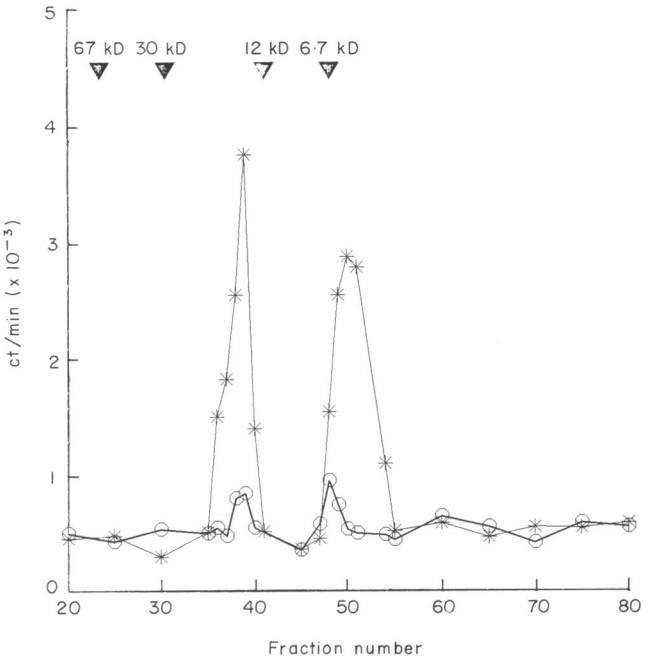

Fig. 4. Sephacryl S 200-SF gel chromatography of whole isolated rat glomeruli-derived IL-1 activity 14 days afer adriamycin injection (*); the same samples after pre-incubation with rabbit anti-IL-1 antiserum (○).

to assess, by the observation of *in vivo* and *in vitro* glomerular IL-1-like cytokine production, the possible contributory role of intrinsic glomerular cells (mainly resident macrophages) in the development of this nephropathy.

With this intention, we have first examined glomerular I-a expression by resident glomerular macrophages during the course of the disease. A previous report [19] showed that in the glomerulopathy induced by aminonucleoside of puromycin, there was first a decrease in I-a expression by lymphohaematopoietic cells and thereafter, an infiltration by blood monocytes and increase in I-a expression by both resident and infiltrating cells. At variance with aminonucleoside nephrosis, in the adriamycin model, there was no glomerular monocyte infiltration, and enhanced display of I-a expression by resident glomerular macrophages was a constant finding during the 4 weeks of this study. At the present moment we do not know the precise mechanism(s) by which changes in glomerular macrophage I-a expression are induced. The absence in the last model of nephrosis of glomerular cell infiltration (the first time point at which animals were killed was at 7 days) can not rule out an early and transient influx of T cell lymphocytes in the glomeruli [20]. It is also possible that adriamycin might induce the release of local mediator factors which could transiently modulate I-a expression in resident macrophages in the setting of glomerular damage [21].

Of particular interest was the observation that whole isolated glomeruli in culture from rats with adriamycin nephrosis secrete an IL-1-like cytokine. Its biologic activity was eluted in two areas corresponding to 10–20 kD and 5–8 kD, closely resembling macrophage IL-1 [7,8].

A concomitance over the course of the experiment between the augmented I-a-bearing glomerular macrophages, increased cytokine secretion and proteinuria was observed, suggesting

that IL-1-like cytokine might be an important factor implicated in the glomerular injury of this model of nephrosis.

We found lower levels (but with mean values higher than controls) of IL-1-like activity in the supernatants from cultured glomeruli in adriamycin rats killed on days 21 and 28 of the disease. At that time, the number of glomerular macrophage-bearing I-a antigens and mean values of proteinuria were also significantly elevated. One interpretation of this observation is that the decreased levels of IL-1-like activity could be secondary to local production of IL-1 inhibitors by glomerular cells, such as uromodulin or other substances [22]. Alternatively, mechanisms exist by which stimuli, directly or indirectly affecting macrophages and/or other intrinsic glomerular cells, can provoke the release of a number of different mediator factors (other than IL-1) with the potential to amplify glomerular capillary wall injury with consequent persistence of proteinuria [2–4].

Taking into account the complex series of interrelated events with interacting cells and mediator systems involved in most situations of glomerular injury, it is difficult to elucidate which cell and a given mediator participate in these reactions. In this sense, we have extended our observation of the capacity of both mesangial cell subpopulations to secrete IL-1-like cytokine through individual long-term cell cultures. Our results have demonstrated that cultured glomerular macrophages, stimulated with appropriate doses of adriamycin, produce significantly higher IL-1-like activity than contractile mesangial cells in culture. Recently, Matsumoto & Atkins [23] have observed in a model of focal and segmental glomerulosclerosis (FGS) induced in the rat by repeated injections of aminonucleoside of puromycin and proteamine sulphate, an increased number of macrophages in the outgrowths from isolated glomeruli in culture. There authors have also demonstrated IL-1 production by macrophages and/or mesangial cells early in the disease process, suggesting a possible pathogenic role of this cytokine in the triggering and development of FGS.

Similarly, our results have demonstrated that in adriamycin nephrosis, IL-1-like cytokine (mainly secreted by resident glomerular macrophages) could also be important in the stuctural and functional disturbance occurring at the glomerular capillary wall level. Nevertheless, and taking into consideration that IL-1 is a pleiotropic factor, eliciting a broad set of immunologic and inflammatory events, one can only speculate on the complexity of the amplification process once this cytokine has been boosted.

Although many problems remain unresolved, the understanding of this polypeptide system network of interactive signals between cells and mediator factors raises the hope of a better knowledge of the mechanisms of injury in this model of nephrosis which bears common features with minimal changes disease in humans.

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