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Cytotoxic effect of autocrine and macrophage-derived nitric oxide on cultured rat mesangial cells

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SUMMARY

Expression of the inducible form of nitric oxide synthase (iNOS) has been found to be up-regulated in cytokine-stimulated mesangial cells (MC) and in experimental glomerulonephritis. Since direct toxicity of nitric oxide (NO) has been implicated in damage of bacteria, neoplastic and intact pancreatic cells, we investigated whether NO is cytotoxic to cultured MC, which may be relevant to pathogenesis of glomerular injury. MC isolated from rat glomeruli generated substantial amounts of nitrite, the stable NO end-product, when cells were stimulated with IL-1β and tumour necrosis factor-alpha (TNF-α). Total DNA synthesis was significantly reduced in the presence of IL-1β and TNF-α, and this effect was completely reversed by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of iNOS. Stimulation of MC with IL-1β and TNF-α caused remarkable toxicity to these cells, measured by the MTT test (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide cleavage, specific cytotoxicity 41·5 $\pm$ 20·3%), and much less prominent MC lysis (^{3}H-thymidine release, specific cytolysis 11·5 $\pm$ 5·3%). Toxic effects of cytokines were fully reversible by the iNOS inhibitor. Lipopolysaccharide (LPS) and interferon-gamma (IFN-γ), but not IL-1β and TNF-α, induced rat peritoneal macrophages to produce large amounts of nitrite. In co-culture, such prestimulated macrophages had significantly cytotoxic (MTT test 62·9 $\pm$ 19·9%) and cytolytic (^{3}H-thymidine release 57·9 $\pm$ 13·8%) effects on MC. Again, this toxicity was totally inhibited in the presence of L-NMMA. We conclude from these results that cytokine-stimulated generation of NO by MC or macrophages is directly toxic to MC, and may play a role in pathogenesis of glomerular injury involving mesangiolysis.

Keywords nitric oxide mesangial cells cytotoxicity cytokines macrophages

INTRODUCTION

Nitric oxide (NO), a labile substance whose biological activity was originally attributed to endothelium-dependent vasorelaxation [1], is now regarded as a highly versatile molecule, acting both as a homeostatic regulator of cellular functions and as a mediator of inflammatory tissue injury [2]. It is synthesized from L-arginine by a family of enzymes, called nitric oxide synthases (NOS), the constitutive type of which (cNOS) generates NO in endothelial cells, platelets and nerves in picomolar concentrations. Production of NO by cNOS, representing homeostatic control mechanisms, in the kidney enables autoregulation of renal blood flow, glomerular filtration and release of renin (reviewed in [3]). On the other hand, the inducible NO synthase (iNOS), expressed in macrophages, vascular smooth muscle cells, mesangial cells and many other cell types, is capable of producing much higher, nanomolar quantities of NO following stimulation with inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), IL-1β and interferon-gamma (IFN-γ) [2]. The activity of iNOS has been linked with macrophage killing of tumour cells [4], parasites and fungi [5] and implicated in the pathogenesis of arthritis [6] and diabetes mellitus [7].

In recent years evidence has accumulated indicating the possible importance of the NO pathway in mediation of experimental immune glomerulonephritis. In this regard Cattell and coworkers have documented production of nitrite (NO_2^-) by glomeruli of nephritic but not normal rats [8] and expression of iNOS in rat immune complex nephritis [9]. Treatment with an inhibitor of iNOS, N^G-monomethyl-L-arginine (L-NMMA), resulted in amelioration of glomerular injury in rat anti-thymocyte serum glomerulopathy [10] and spontaneous lupus nephritis in mice [11]. Given the evidence on a NO-dependent cytolysis of microbes [5], tumour [4] and intact pancreatic islet cells [7] by macrophages and an antiproliferative effect of NO donors on mesangial cell cultures [12], we investigated a possibility that cytokine-induced NO production by macrophages and mesangial cells (MC) may be directly toxic to these MC *in vitro*, thus potentially contributing to pathogenesis of immune glomerular injury.

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MATERIALS AND METHODS

Animals

Glomerular MC and peritoneal macrophages were obtained from male Sprague Dawley rats, weighing 200–250 g, purchased from Charles River (Sulzfeld, Germany).

Reagents

Human recombinant IL-1β and basic fibroblast growth factor (bFGF) were purchased from Boehringer (Mannheim, Germany), TNF-α was obtained from BASF/Knoll (Ludwigshafen, Germany). Thioglycolate medium, L-NMMA, chemicals used for the nitrite estimation, MTT, IFN-γ and lipopolysaccharide (LPS) were all purchased from Sigma (Deisenhofen, Germany). Radioactive products were from Amersham Buchler (Braunschweig, Germany). Tissue culture plasticware was from Falcon (Becton Dickinson, Heidelberg, Germany), media and sera from GIBCO-BRL (Eggenstein, Germany).

Mesangial cell preparation and cell culture

For the preparation and culture of glomerular MC from rats, standard techniques were used that were reported previously [13]. The cells were characterized as MC by immunocytochemical staining for Thy1.1, smooth muscle cell actin and myosin and showed a smooth muscle cell-like morphology. MC were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO-BRL), 2 mM glutamate, 5 ng/ml insulin, 100 U/ml penicillin and 1 mg/ml streptomycin (all from Sigma). Cells were used for experiments at subconfluence or passaged at confluence with trypsin/EDTA (0·05%/0·02% (w/v)). To obtain nearly quiescent cells, MC were maintained in 0·5% FCS-containing medium for 4 days before cytokine treatment. Cells were used between passages 8 and 19.

^{3}H-thymidine incorporation

Total DNA synthesis was estimated on the basis of ^{3}H-thymidine incorporation into the cell genome. Tests were performed on quiescent mesangial cultures grown in 96-well plates as described above (20×10^3 cells/well in 0·2 ml medium). Cells were stimulated for 24 h with TNF-α (10 ng/ml) and IL-1β (25 U/ml) with or without L-NMMA (1 mM) in DMEM containing 0·5% FCS. In parallel, the same additions were used in cell cultures stimulated to grow by bFGF (10 ng/ml). Cells incubated in DMEM–0·5% FCS with or without bFGF served as respective controls. Subsequently, cytokines and L-NMMA-containing media were discarded and cells labelled for 6 h with ^{3}H-thymidine (2·5 μCi/ml) in DMEM supplemented with 10% FCS or bFGF. Radioactive medium was removed, cells washed twice with PBS, collected with an automatic harvester (Cambridge Technology, Watertown, MA) and their radioactivity measured with a liquid scintillation system (Beckman, Munich, Germany). Results were expressed as ct/min (mean values $\pm$ s.d. of at least six samples per assay).

Incorporation of ^{3}H-thymidine following stimulation of cell growth by bFGF was $\approx$ 76% of that obtained with 10% FCS.

MTT test

Quiescent rat mesangial cells, grown in 96-well plates, were washed three times with sterile PBS and incubated for 72 h in DMEM–0·5% FCS, in the presence or absence of TNF-α (10 ng/ml) and IL-1β (25 U/ml), with or without L-NMMA (1 mM). Thereafter, media were discarded, cells washed twice with PBS and their potential to cleave MTT as a measure of viability was assessed according to the method described by Ferrari *et al.* [14], with some modifications. MTT (20 μl) dissolved in PBS (5 mg/ml) was added to each well of a 96-well plate and incubated for 3 h at 37°C. After discarding supernatants, 0·1 ml of 96% ethanol was added to dissolve formazan crystals and optical density (OD) measured with 570 nm wavelength and 690 nm reference. Data were expressed as OD (mean $\pm$ s.d. of at least six samples) or percentage of specific cytotoxicity: percent C = (spontaneous OD − experimental OD)/spontaneous OD $\times$ 100.

^{3}H-thymidine release assay

Cytolysis of mesangial cells was quantified by measuring radioactivity of ^{3}H-thymidine liberated from labelled cells during 72 h coculture with cytokines, L-NMMA and/or prestimulated rat peritoneal macrophages. In the series of preliminary experiments 72 h incubation was found optimal for assessment of ^{3}H-thymidine release: radioactivity of supernatants rising from the onset up to this time point, assuming a plateau thereafter (data not shown). Quiescent rat MC, grown in 96-well plates as described above, were labelled with ^{3}H-thymidine (0·2 μCi/10^3 cells) during 48 h culture in medium containing 10% FCS. Thereafter, radioactive medium was discarded, cells were washed three times with sterile PBS and incubated for a further 72 h in DMEM–0·5% FCS, in the presence or absence of TNF-α (10 ng/ml) and IL-1β (25 U/ml), with or without L-NMMA (1 mM). After 72 h incubation, 0·1-ml aliquots of culture supernatants were assessed for radioactivity of ^{3}H-thymidine in a liquid scintillation counter. Maximal release of the ^{3}H-thymidine from labelled MC was assessed by measuring radioactivity of 0·1-ml aliquots of culture supernatants after lysing cells with 5% Triton X-100 for 30 min. Results were expressed as ct/min (mean $\pm$ s.d. from at least six wells) or percentage cytolysis, calculated using the formula: percent C = (experimental release − spontaneous release)/(maximal release − spontaneous release) $\times$ 100.

Macrophage-mediated cytotoxicity of MC

Rat macrophages were recovered from peritoneal lavage fluid of animals challenged intraperitoneally 4 days before with 8 ml of 4% thioglycolate medium. Peritoneal exudate cell suspension, consisting of $\approx$70–90% macrophages, as judged by morphologic criteria of Giemsa-stained samples, was washed three times with DMEM–10% FCS and incubated for 2 h at 37°C in plastic Petri dishes. Thereafter, non-adherent cells were washed off with warm (37°C) PBS. Remaining macrophages (2×10^6 cells per Petri dish, 10 cm diameter) were stimulated with IL-1β and TNF-α, at concentrations specified above for mesangial cells, or with IFN-γ (100 U/ml) and LPS (10 μg/ml) for 24 h in DMEM–10% FCS, in the presence or absence of L-NMMA (1 mM). After stimulation, conditioned media were discarded, cells detached from plastic by rinsing with cold (4°C) PBS, containing 10 mM EDTA pH 7·5, washed twice with DMEM–10% FCS and seeded in 96-well plates containing quiescent MC, at 1:1 mesangial cell:macrophage ratio (2×10^3 macrophages/well). Following 72 h coculture, the MTT test was performed as described above and results expressed as OD or percentage of specific cytotoxicity: percent C = 100 − (OD macrophages + mesangial cells) − (OD macrophages)/(OD mesangial cells) $\times$ 100.

For experiments assessing cytolysis, macrophages were added to wells containing MC prelabelled with ^{3}H-thymidine (1:1) and after 72 h supernatants were harvested and their radioactivity was measured as described above for the ^{3}H-thymidine release assay. Specific cytolysis of MC by macrophages was calculated using the formula: percent cytolysis = (release by macrophages – spontaneous release)/ (maximal release – spontaneous release) × 100.

Nitrite estimation in conditioned medium

For the above cytotoxicity experiments, MC or 24 h macrophage cultures, stimulated with cytokines and/or treated with L-NMMA, were grown in parallel in phenol red-free culture medium (Petri dishes, 3 cm diameter) for 24 h or 72 h. The nitrite content in supernatants was measured with the Griess method [15]. Briefly, 250 μl conditioned medium as well as medium containing known sodium nitrite contents (controls) were mixed with 50 μl Griess reagent (25 mM sulphanilamide and 25 mM naphthylethylenediamine) and 25 μl 6 M HCl for 30 min. Thereafter, OD was measured at 540 nm and the nitrite content of conditioned medium calculated from the standard curve obtained from controls.

Statistical analysis

After verifying normal distribution of data, significance of differences between mean values in individual groups was evaluated using Student's *t*-test. $P < 0{\cdot}05$ was regarded as significant. All data were expressed as means ± s.d.

RESULTS

Effect of endogenously produced NO on ^{3}H-thymidine uptake by MC

We (Beck *et al.* [16]) and others [17] have previously reported that co-stimulation of cultured mesangial cells with IL-1β and TNF-α results in generation of greater quantities of nitrite than when cells are stimulated with either cytokine alone. In the present experiments triggering quiescent mesangial cells with IL-1β and TNF-α caused a remarkable increase in nitrite production, inhibitable by L-NMMA as depicted in Fig. 1. A comparable induction of NO production by LPS and IFN-γ (concentrations indicated above for stimulation of macrophages, data not shown) was seen in MC in accordance with a previous report from another group [18]. Stimulation of cell growth with bFGF brought about only a minor elevation in basal NO_2^- (to $46{\cdot}67 \pm 9{\cdot}98$ nM/10^6 cells) and a lower increase in nitrite production following treatment with IL-1β and TNF-α (to $79{\cdot}75 \pm 17{\cdot}02$ nM/10^6 cells), than that observed in quiescent cells.

Cultures of renal mesangium pretreated with IL-1β and TNF-α in the way demonstrated above to generate large quantities of nitrite, incorporated significantly less ($P < 0{\cdot}0005$) radioactive tracer than did untreated controls (Fig. 1). This inhibition of MC DNA synthesis was blocked in the presence of L-NMMA (Fig. 1), while L-NMMA alone did not modify uptake of ^{3}H-thymidine by untreated cells ($18\,140 \pm 3667$ ct/min, $n = 5$). This effect was also evident in cells stimulated to grow with bFGF (Fig. 1).

NO-dependent MC toxicity

Treatment with IL-1β and TNF-α remarkably ($P < 0{\cdot}0005$) reduced the viability of quiescent MC, and this effect was totally abolished by addition of L-NMMA, as illustrated in Fig. 2. Pooled data from three identical experiments revealed specific cytotoxicity of IL-1β and TNF-α ($41{\cdot}5 \pm 20{\cdot}3\%$), also completely inhibitable by L-NMMA. Cells stimulated to proliferate with bFGF were even more sensitive to toxicity of NO generated by cytokines (Fig. 2), with specific cytotoxicity of $80{\cdot}2 \pm 14{\cdot}1\%$, reduced to $14{\cdot}6 \pm 2{\cdot}3\%$ by L-NMMA (data from one experiment).

Quiescent MC, prelabelled with ^{3}H-thymidine, released in 72 h culture with IL-1 and TNF-α significantly more of the radioactive tracer than did control cells, cultured in parallel in the absence of cytokines (IL-1β and TNF-α $19\,132 \pm 911$ ct/min; $n = 6$; control $16\,581 \pm 1818$ ct/min; $n = 6$; $t = 3{\cdot}07$, $P \leqslant 0{\cdot}01$). Addition of L-NMMA completely prevented cytolysis caused by NO-generating cytokines (L-NMMA, IL-1β and TNF-α $16\,657 \pm 3002$ ct/min, $n = 6$). The cytolytic effect of IL-1β/TNF-α-generated NO in quiescent cells was subsequently confirmed by three separate experiments, pooled results of which indicate $11{\cdot}5 \pm 5{\cdot}3\%$ of specific cytolysis, totally abrogated on each occasion by the L-NMMA. Specific cytolysis of MC stimulated to grow with bFGF was even less evident, amounting to $8{\cdot}2 \pm 0{\cdot}6\%$, also inhibitable with L-NMMA.

Production of nitrite by cytokine-stimulated peritoneal macrophages

Peritoneal macrophages elicited *in vivo* with thioglycolate produced in 24 h culture $20{\cdot}5 \pm 4{\cdot}5$ nM $NO_2^-/10^6$ cells, while addition of L-NMMA almost completely inhibited this production (Fig. 3). Stimulation with IL-1β and TNF-α did not result in any appreciable increase in basal nitrite production by these cells. As summarized in Fig. 3, triggering macrophages with LPS and IFN-γ remarkably enhanced generation of NO_2^- ($P < 0{\cdot}0005$), inhibitable by the L-NMMA.

Toxicity of LPS/IFN-γ-triggered macrophages to MC

Coculture of quiescent MC with macrophages, elicited *in vivo* by thioglycolate, resulted in significant toxicity to MC, determined by the MTT test (OD: control mesangial cells, 307 ± 66, $n = 28$; macrophages, 180 ± 49, $n = 5$; $t = 5{\cdot}08$, $P < 0{\cdot}0005$), although this effect was not inhibitable by L-NMMA (OD: 153 ± 18, $n = 5$; OD macrophages, L-NMMA *versus* OD macrophages, $t = 1{\cdot}17$, NS). Similarly, reduced target cell viability was observed, when macrophages were preincubated with IL-1β and TNF-α, but treatment with L-NMMA did not significantly modify this effect (OD: IL-1β and TNF-α-pretreated macrophages, 193 ± 53, $n = 5$; IL-1β/TNF-α/L-NMMA, 229 ± 65, $n = 8$; $t = 1{\cdot}09$, NS). On the other hand, pretreatment of macrophages with cytokines, shown in parallel to generate substantial quantities of nitrite, LPS and IFN-γ, resulted in a pronounced cytotoxicity against MC (OD: LPS and IFN-γ-pretreated macrophages, 114 ± 36, $n = 7$; difference with control mesangial cells: $t = 10{\cdot}4$, $P < 0{\cdot}0005$). This cytotoxicity was significantly (by ≈31%), inhibitable by L-NMMA (OD: LPS/IFN-γ/L-NMMA: 210 ± 53, $n = 5$; difference with LPS, IFN-γ: $t = 3{\cdot}56$, $P < 0{\cdot}005$). Figure 3b depicts specific cytotoxicities of cytokine- and LPS-pretreated macrophages against quiescent MC.

Likewise, triggering of macrophages with LPS and IFN-γ, strongly stimulating NO_2^- production, markedly elevated release of ^{3}H-thymidine by cocultured MC (macrophages stimulated with LPS and IFN-γ, 5916 ± 1427 ct/min; control, 3662 ± 385 ct/min; $n = 8$; $t = 4{\cdot}17$, $P \leqslant 0{\cdot}0005$; specific cytolysis $57{\cdot}9 \pm 13{\cdot}8\%$), and this effect was notably preventable by L-NMMA (macrophages, LPS, IFN-γ and L-NMMA: 4126 ± 707, $n = 6$; difference with control values: $t = 1{\cdot}45$, $P \leqslant 0{\cdot}1$, NS).

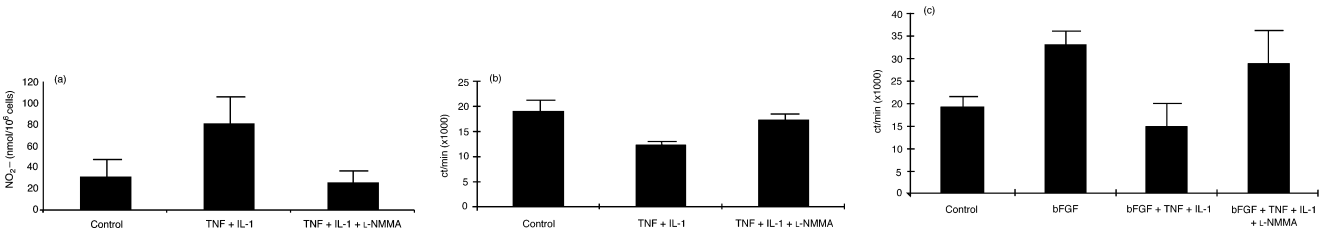

Fig. 1. Mean values and s.d. (a) Generation of nitrite by mesangial cell cultures upon stimulation with IL-1β and TNF-α significantly exceeded control values ($P < 0{\cdot}0005$). In the presence of N^G-monomethyl-L-arginine (L-NMMA) this effect was abrogated. (b) Results of the ^{3}H-thymidine uptake test in quiescent mesangial cells. Incorporation of thymidine was significantly reduced ($P < 0{\cdot}0005$) in cells stimulated with IL-1β and TNF-α, and this effect was reversed by the inhibitor of the inducible NO synthase (iNOS), L-NMMA. (c) DNA synthesis in mesangial cells proliferating in response to basic fibroblast growth factor (bFGF) was remarkably reduced ($P < 0{\cdot}0005$) by treatment with the nitrite-eliciting combination IL-1β and TNF-α, and this effect was abolished by addition of L-NMMA.

DISCUSSION

Stimulation of quiescent MC with IL-1β and TNF-α resulted in a notable increase in nitrite production compared with basal generation of NO_2^- by these cells. The ability of MC to enhance production of nitrite and expression of the iNOS in response to these proinflammatory cytokines have been reported by many groups, including ourselves [15–17]. Results of some of these studies [16,17], by indicating that co-stimulation of mesangium with IL-1β and TNF-α acts synergistically to generate large amounts of nitrite, prompted us to utilize concurrently both cytokines in our experiments. MC stimulated to proliferate with bFGF did not in our hands respond as vigorously by production of nitrite to stimulation with IL-1β and TNF-α as did quiescent cells, contrary to vascular smooth muscle cells, which more remarkably enhance generation of NO_2^- upon triggering with IL-1α and bFGF, as described by Scott-Burden *et al.* [19]. This difference in reactivity to proinflammatory cytokines may indicate that MC, as specialized glomerular pericytes, may have a greater potential to secrete NO in response to inflammatory stimuli than their less differentiated counterparts in blood vessels of higher calibre.

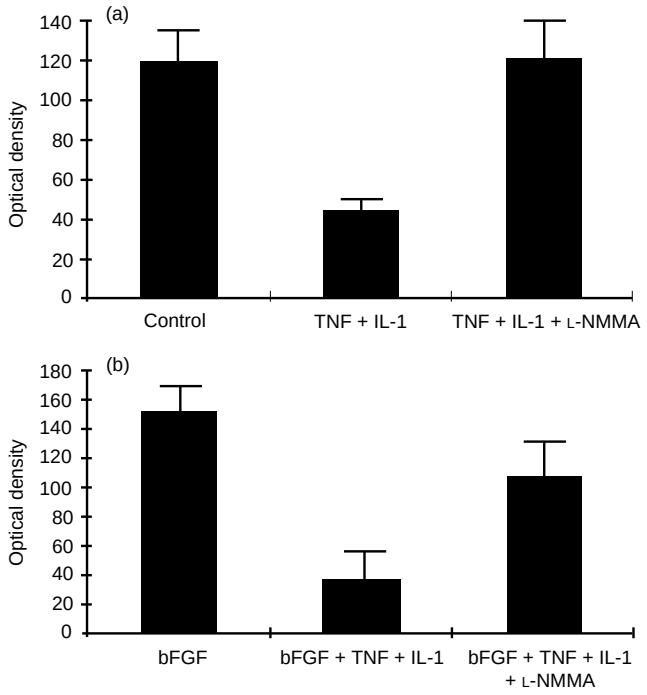

Fig. 2. Cytotoxicity of IL-1β- and TNF-α-treated mesangial cells, assessed by the MTT test (means $\pm$ s.d.). Addition of nitrite-generating cytokines markedly reduced viability of both quiescent ((a), significance $P < 0{\cdot}0005$) and basic fibroblast growth factor (bFGF)-stimulated ((b), significance $P < 0{\cdot}0005$) cells, and this cytotoxicity was inhibited by N^G-monomethyl-L-arginine (L-NMMA).

A possibility that locally produced NO may play a role in pathogenesis of glomerular injury owing to its cytotoxic potential has been postulated [2,9], although direct evidence is still lacking. Garg & Hassid have documented inhibition of mesangial proliferation by NO donor vasodilators, but cell viability was not adversely affected by NO in their study [12]. Further down this line, Scott-Burden *et al.* reported [19] that levels of nitrite released by vascular smooth muscle cells in response to IL-1α correlated with the inhibition of thymidine incorporation. On the other hand, in a recent report Mohaupt *et al.* [20] demonstrated lack of significant antiproliferative effect of autocrine (IL-1β-stimulated), or exogenous NO on cultured rat MC. Our results, indicating reduced thymidine uptake by mesangial cultures stimulated with NO-generating IL-1β and TNF-α, are not in disagreement with that study, for a number of reasons. First, our experiments were designed to examine the effect of high concentrations of endogenous, or macrophage-derived nitrite on MC, in a manner mimicking conditions of acute inflammatory milieu, exposing cells to combined action of high concentrations of cytokines such as IL-1β (25 U/ml) and TNF-α (10 ng/ml), while Mohaupt *et al.* employed only IL-1β at much lower concentrations (5 U/ml). Second, in the cited study cell proliferation was induced by 5% FCS or platelet-derived growth factor (PDGF), both regarded as blockers of iNOS induction on the transcriptional level (FCS, K.F. Beck, unpublished results; PDGF, [21]). As a result, MC cultures in our experiments were exposed to over three times higher NO concentrations than was the case in the paper of Mohaupt *et al.* Moreover, reduced thymidine incorporation observed in our study might not have been due to direct antimitogenic influence of nitrite, but rather to its cytotoxicity to the MC, since the level of radioactivity of IL-1β- and TNF-α-stimulated cells was much lower than that of control, unstimulated cultures (Fig. 1). We have also noted that MC treated with NO-generating cytokines showed an alteration of morphology suggestive of early necrotic changes (data not shown). The deleterious effect exerted by NO on MC was even more evident in cells stimulated to proliferate with bFGF.

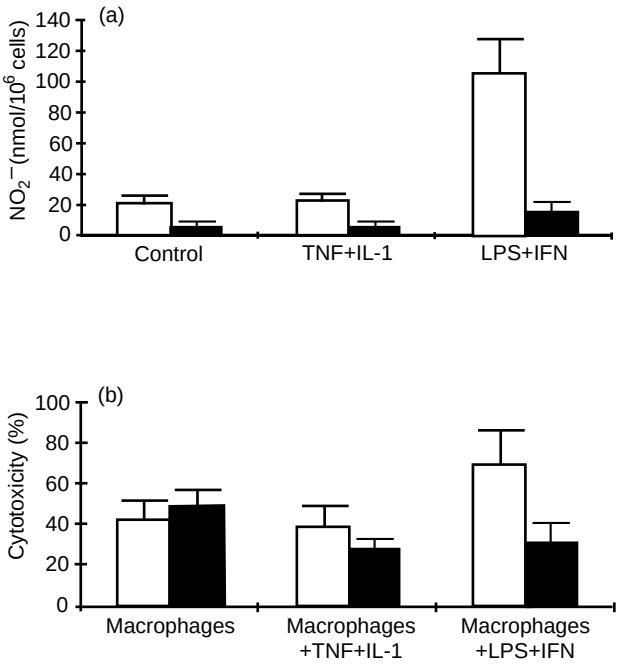

Fig. 3. (a) Production of nitrite by rat peritoneal macrophages triggered with cytokines and lipopolysaccharide (LPS). The combination of LPS and IFN-γ notably enhanced generation of nitrate ($P < 0{\cdot}0005$), which was not the case with IL-1β/TNF-α. (b) Specific cytotoxicity of cytokine-stimulated macrophages against mesangial cells, assessed by the MTT test. High cytotoxicity of the LPS/IFN-γ-treated macrophages was significantly ($P < 0{\cdot}005$) reduced by the inducible NO synthase (iNOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA), while these differences were not significant in the case of control and IL-1β/TNF-α-stimulated macrophages. Error bars represent s.d. □, Without L-NMMA; ■, with L-NMMA.

Suppression of DNA synthesis, seen in both quiescent and proliferating cells, prompted us to assess the direct cytotoxic potential of IL-1β- and TNF-α-induced nitrite against MC, using MTT tests. The concept of mesangial damage by humoral inflammatory mediators is not new: one of us (Z.H.) with a group from McGill University has demostrated the ability of recombinant TNF-α to cause lysis of cultured MC [22]. Since this cytokine has also been documented to be secreted by MC upon stimulation with LPS [23], it appears that under inflammatory conditions it may act as a mediator of self-destruction, and this mechanism may equally operate with respect to the L-arginine/NO pathway. Cytotoxic potential of NO against various cell types has been documented, including microbial [5], transformed [4] and intact somatic cells [7]. We have therefore been hardly surprised to see the results of current experiments on MC toxicity, determined by the MTT test. This method, based on the capacity of mitochondrial enzymes of viable cells to transform the MTT into blue formazan, compares favourably with the thymidine uptake test in terms of sensitivity of measuring cytotoxic cell damage, and has been found more sensitive in this regard than the thymidine release assay measuring cytolysis. This difference results from the fact that the radioisotope release test measures only cytolysis of target cells, while the MTT method reflects both cytotoxic and cytolytic effects [14]. Our data support the idea of a NO-mediated mesangiotoxicity in anti-thymocyte serum nephritis raised by Narita *et al.* [10]. Their report has documented almost total (90%) prevention of MC lysis, previously attributed to activation of complement, by treatment of rats with L-NMMA before administration of nephrotoxic antibody. Other effects of iNOS inhibitor included reduction of proteinuria, extracellular matrix accumulation and urinary nitrite excretion, and were reproduced, along with abrogation of mesangiolysis, by dietary L-arginine restriction. Interestingly, a recent publication of Waddington *et al.* [24] claimed the opposite effect of iNOS inhibition in rat nephrotoxic nephritis. L-arginine depletion with arginase exacerbated glomerular injury in the early stage of this nephropathy, indicating a protective potential of NO against development of inflammatory lesions. Therefore, the significance of activation of the L-arginine/NO pathway remains to be established in various models of glomerulonephritis, representing different pathogenic mechanisms.

Peritoneal macrophages responded vigorously to LPS and IFN-γ by secretion of nitrite, and these results support previous reports claiming a resemblance between mesangial and macrophage iNOS [25]. On the other hand, in our experiments macrophage iNOS was unresponsive to IL-1β and TNF-α, as opposed to the MC NO synthase. It is noteworthy that a combination of LPS and IFN-γ has been demonstrated to induce selectively production of NO by murine peritoneal macrophages, contrary to zymosan and IFN-γ, that caused generation of both superoxide and NO by these cells [26]. Our data also point at macrophages as yet another potential source of NO released and acting at the glomerular level to mediate inflammatory damage to the cells of renal tuft. Preincubation of macrophages with the LPS and IFN-γ, initiating profuse secretion of nitrite, brought about significant cytotoxicity of mesangial cells ($62{\cdot}9 \pm 19{\cdot}9\%$), and this activity was only partially, though significantly, suppressed by L-NMMA (by 31%). This partial inhibition is indicative of other macrophage-derived factors toxic to MC, aside from the L-arginine/NO pathway. Moreover, such factors might have been released by macrophages prestimulated with the IL-1β and TNF-α, a combination unable to raise NO production in these cells, since their co-incubation with MC resulted in 37·2% of specific toxicity, not significantly affected by the L-NMMA. It is conceivable that these mesangiotoxic factors may include superoxide, because this oxygen radical has been found secreted in abundance by macrophages isolated from glomeruli of rats with anti-thymocyte nephritis, featuring mesangial necrosis in the early phase of the disease [27]. Triggering *in vivo* of macrophages with thioglycolate without subsequent cytokine stimulation *in vitro*, apparently generated their cytotoxic activity against cocultured MC, as indicated by positive results of the MTT test. Nonetheless, mesangiotoxicity of thioglycolate-elicited and not LPS/IFN-γ-stimulated macrophages was clearly NO-independent, since treatment with L-NMMA did not affect viability of target cells. The importance of monocytes/macrophages in pathogenesis of glomerulonephritis has been long recognized, and it has been suggested that infiltrating mononuclear phagocytes, but not resident MC, are the main source of NO produced in glomeruli of rats with experimental nephritis [8]. The authors of the latter article claimed inability of MC to produce nitrite in culture, although they have not used any combination of cytokines to stimulate MC iNOS. Our data indicate a potential of both cell types to contribute to the NO-dependent glomerular injury in response to inflammatory cytokines.

Quiescent MC treated with cytokines demonstrated only a minor degree of cytolysis in response to the autocrine NO, as assessed with the ^{3}H-thymidine release method ($11{\cdot}5 \pm 5{\cdot}3\%$), whereas specific cytotoxicity (MTT test) was much higher under these circumstances ($41{\cdot}5 \pm 20{\cdot}3\%$). This result may be indicative of a rather low extent of direct cell disintegration, compared with severe metabolic impairment accounting for cytotoxicity with no structural damage in autocrine NO-mediated MC injury, even though the MTT test measuring cytotoxicity reflects both toxic and lytic damage to the cells. It is noteworthy that this difference was not significant in the case of macrophage-dependent mesangiolysis/toxicity ($57{\cdot}9 \pm 13{\cdot}8\%$ *versus* $62{\cdot}9 \pm 19{\cdot}9\%$); lower quantities of NO generated by MC than by macrophages may at least in part account for this discrepancy.

Cytotoxicity of NO is mediated by three main mechanisms: interference with mitochondrial electron transport chain [28], reaction with superoxide to form peroxynitrite, a source of toxic hydroxyl radicals [29], and induction of alterations in target cell genome [30]. The first mechanism, acting through inactivation of respiratory enzymes by depleting them of iron, could be implicated in the cytotoxic effect disclosed in our study by the MTT method, detecting abnormalities in mitochondrial respiration. Finally, NO-mediated fragmentation of DNA could under some circumstances occur in a form of programmed cell death, or apoptosis [31], and the importance of this mechanism to the pathogenesis of renal diseases is presently being investigated in our laboratory.

In conclusion, our results provide evidence of cytotoxic damage to MC by NO, a molecule long recognized as being only a homeostatic mediator, and indicate its potential importance in pathogenesis of immune/inflammatory glomerular injury.

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