Clin Exp Immunol 1997; **108**:318–323

Up-regulation of macrophage colony-stimulating factor (M-CSF) and migration inhibitory factor (MIF) expression and monocyte recruitment during lipid-induced glomerular injury in the exogenous hypercholesterolaemic (ExHC) rat

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(Accepted for publication 23 January 1997)

SUMMARY

Although macrophages play an important role in lipid-induced glomerular injury, we know little of the mechanisms by which hyperlipidaemia induces monocyte recruitment. This study investigated the role of M-CSF and macrophage MIF in monocyte recruitment during the development of lipid-induced glomerular injury in the susceptible ExHC rat strain. Groups of five ExHC rats were fed a high cholesterol diet (HCD) containing 3% cholesterol, 0·6% sodium cholate and 15% olive oil, and killed after 3 days, 1, 2 or 6 weeks. Control animals were killed on day 0 or after 6 weeks on a normal diet. Animals were hypercholesterolaemic 3 days after the induction of the HCD, but showed no change in plasma triglycerides over the 6-week period. Glomerular macrophage accumulation was first evident at 1–2 weeks and increased up to week 6, when macrophage-derived foam cells were seen in almost all glomeruli, and segmental lesions and mild proteinuria were also evident. Combined *in situ* hybridization and immunohistochemistry staining demonstrated that, coincident with the induction of hypercholesterolaemia on day 3, there was marked up-regulation of M-CSF and MIF mRNA expression by intrinsic glomerular cells (mostly mesangial cells and podocytes) which preceded monocyte recruitment. There was a highly significant correlation between the number of M-CSF and MIF-positive cells and glomerular macrophage accumulation over the 6-week period. Although some glomerular macrophages and foam cells exhibited M-CSF and MIF expression, the major source of these molecules was intrinsic glomerular cells. No local macrophage proliferation was observed during the development of glomerular lesions. In conclusion, hypercholesterolaemia caused marked up-regulation of M-CSF and MIF expression by intrinsic glomerular cells, which correlated with monocyte recruitment and the development of lipid-induced glomerular injury. This is the first study to implicate local synthesis of MIF in the pathogenesis of lipid-induced lesions.

Keywords macrophage colony-stimulating factor macrophage migration inhibitory factor glomerulonephritis cholesterol *in situ* hybridization

INTRODUCTION

Hyperlipidaemia has been shown to accelerate the induction and progression of renal injury leading to glomerular sclerosis [1,2]. Lipid-induced glomerular injury is thought to be mediated in part by macrophages, on the basis that glomerular macrophage accumulation is a feature of animal models of endogenous and diet-induced hyperlipidaemia, and macrophage depletion inhibits lipid-induced renal injury [3–6]. However, we know little of the mechanisms by which hyperlipidaemic states induce macrophage accumulation within the glomerulus.

Given the similarities between the development of atherosclerotic lesions and cellular immune responses [7], we have investigated the role of M-CSF and macrophage MIF in macrophage accumulation in lipid-induced glomerular injury. M-CSF is the main growth factor for monocyte production in bone marrow, is

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a potent chemotactic molecule for monocytes and can prime or directly stimulate a range of monocyte/macrophage functions [8]. Although M-CSF has not been studied in lipid-induced renal injury, several studies point to a role for this molecule in monocyte recruitment in this form of renal injury. Local M-CSF expression and macrophage accumulation have been demonstrated in vascular atherosclerotic lesions [9–11]. Increased glomerular M-CSF expression has been implicated in glomerular macrophage accumulation and the development of glomerular injury in murine lupus nephritis [12]. In addition, local macrophage proliferation is a major mechanism of macrophage accumulation in some severe forms of immunologically induced kidney injury [13,14], which may be driven by local M-CSF production.

MIF is a potent proinflammatory mediator which is produced by many cell types, including macrophages and resident kidney cells [15]. It is required for macrophage accumulation in the skin DTH response and is a counter-regulator of glucocortoid action [16,17]. We have recently demonstrated a dramatic up-regulation of renal MIF expression in association with macrophage infiltration and the development of glomerular lesions in a rat model of crescentic glomerulonephritis [18]. However, it is not known whether MIF may contribute to macrophage recruitment in the development of lipid-induced renal injury. Therefore, this study examined glomerular M-CSF and MIF expression, together with macrophage accumulation, in ExHC rats fed a high cholesterol diet—a model of lipid-induced glomerular injury.

MATERIALS AND METHODS

Experimental model

ExHC rats are derived from the Sprague-Dawley strain and develop marked hypercholesterolaemia without any increase in serum triglyceride levels when fed a cholesterol-supplemented diet [19]. Twenty-five male ExHC rats weighing 150 g (generously supplied by Takeda Chemical Industries Ltd, Osaka, Japan) were placed on a normal diet supplemented with 3% (w/w) cholesterol, 0·6% (w/w) sodium cholate and 15% (w/w) olive oil (high-cholesterol diet (HCD); obtained from Clea Inc., Osaka, Japan), as previously described [5]. Groups of five rats were killed on day 3 or weeks 1, 2 and 6 after the start of the HCD. As a control, one group of five rats was killed before the start of the HCD (designated day 0) and one group was killed after being fed for 6 weeks on a normal diet (ND; CE-2; obtained from Clea Inc.).

Serum lipids and renal injury

Protein levels in 24 urine collections made before killing animals were measured by the coommassie dye method (BioRad Labs, Richmond, CA). Plasma levels of total cholesterol, triglycerides, creatinine and albumin as well as haematocrit and leucocyte counts were determined by standard methods.

Histology

Paraffin sections of tissues fixed in 10% buffered formalin were stained with period acid-Schiff reagent and periodic acid-silver methenamine. Glomerular segmental lesions were assessed on 150 glomerular cross-sections per animal, as previously described [5].

Probes

Probes used were: a 706-bp fragment of rat M-CSF cDNA prepared by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into pBluescript SK + (Stratagene, La Jolla, CA) [20]; and a 420-bp fragment of mouse MIF cDNA in pBluescript SK + (Stratagene) [21]. Sense and anti-sense cRNA probes were synthesized from linearized plasmids using T7 or T3 polymerases and a nucleotide mix containing digoxigenin (Dig)-11-UTP according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Probes were precipitated and Dig incorporation was assessed by dot blot [22].

Antibodies

Mouse MoAbs used were: ED1, anti-CD68, monocytes and macrophages [23]; mouse anti-human MIF MoAb which recognizes murine and rat MIF [15]; PC10, anti-proliferating cell nuclear antigen [24] (Dakopatts, Glostrup, Denmark). Peroxidase- and alkaline phosphatase-conjugated goat anti-mouse IgG, mouse peroxidase anti-peroxidase complexes, and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes were purchased from Dakopatts.

Combined in situ *hybridization and immunohistochemistry staining*

Detection of mRNA within tissue sections by *in situ* hybridization used a new microwave-based protocol [18,25]. Formalin-fixed 4-μm paraffin sections were dewaxed, immersed in 0·01 M sodium citrate pH 6·0 and treated with two rounds of 5 min heating in a microwave oven (2450 MHz at 800 W), washed in PBS, incubated in 0·2 M HCl for 10 min, washed in PBS, incubated in 1% Triton X-100 for 10 min, washed in PBS and then digested for 20 min with 10 μg/ml proteinase-K (Boehringer) in 0·5 M NaCl, 10 mM Tris–HCl pH 8·0 at 37°C. After tissue pretreatment, sections were washed in 2 × SSC, prehybridized for 1 h and then hybridized overnight at 42°C with 0·3 μg/ml Dig-labelled probe in 50% deionized formamide, 4 × SSC, 2 × Denhardt's solution, 1 mg/ml salmon sperm DNA, 1 mg/ml yeast tRNA in 50 mM Tris–HCl pH 7·0. Sections were washed twice in 2 × SSC at room temperature and then in 0·1 × SSC at 42°C. To detect the bound probe, sections were preincubated with 10% fetal calf serum (FCS) and 10% normal sheep serum in PBS for 20 min, drained, incubated with alkaline phosphatase-conjugated sheep anti-Dig IgG (Boehringer) for 60 min, washed in PBS, developed in the dark with NBT and X-phosphate (Boehringer) in a buffer containing 2 mM levamisole for 3–4 h to produce a purple/brown colour and then washed in water. Sense cRNA probes, labelled to the same specific activity as anti-sense cRNA probes, gave no hybridization signal.

Immunostaining was performed following *in situ* hybridization, as previously described [18,26]. Sections were treated with microwave oven heating, as described above, preincubated with 10% FCS and 10% goat serum in PBS for 20 min, drained and labelled with the ED1 or anti-MIF MoAb for 60 min. Sections then were washed in PBS, incubated sequentially with alkaline phosphatase-conjugated goat anti-mouse IgG and mouse APAAP complexes and developed with fast blue BB base (Sigma Chemical Co., St Louis, MO) to produce blue colour. Sections were mounted in aqueous medium. Controls used an isotype-matched irrelevant primary MoAb.

Local macrophage proliferation was assessed by ED1 MoAb labelling of paraffin sections by three-layer immunoperoxidase, followed by microwave treatment and then labelling with the PC-10 MoAb using three-layer immunoalkaline phosphatase, as previously described [26].

Quantification of glomerular cell populations

Combined *in situ* hybridization and immunohistochemistry

staining were quantified. The number of single- and double-stained cells were counted under high power (×400) in 20 glomerular cross-sections per animal on coded slides. Data are shown as the mean ± 1 s.d. (mean ± s.d.).

Statistical analysis

Differences between glomerular cell populations were analysed by one-way analysis of variance (ANOVA) using the Tukey–Kramer multiple comparison test. The Pearson single correlation coefficient was used to compare different glomerular cell populations over the entire disease course.

RESULTS

Lipid-induced renal injury

Animals on the HCD showed no differences in body weight, kidney weight, blood pressure, leucocyte count, haematocrit, plasma albumin or plasma creatinine from rats fed a normal diet for 6 weeks (data not shown). ExHC rats fed the HCD rapidly developed significant hypercholesterolaemia (41 ± 3, 190 ± 14, 226 ± 25, 423 ± 36 and 362 ± 27 mg/dl on days 0, 3 and weeks 1, 2 and 6, respectively; all $P < 0{\cdot}01$ *versus* day 0) and mild proteinuria at week 6 ($21{\cdot}9 \pm 2{\cdot}7$ *versus* $4{\cdot}3 \pm 0{\cdot}4$ mg/24 h on day 0; $P < 0{\cdot}01$), but maintained normal levels of plasma triglycerides. Control ExHC rats fed a normal diet showed no changes in plasma cholesterol, plasma triglycerides or urinary protein excretion.

Glomerular histology

ExHC rats fed a normal diet for 6 weeks had a normal kidney histology. In contrast, ExHC rats fed an HCD developed characteristic glomerular lesions which have been described in detail previously [22]. Briefly, foam cells and/or mesangial cells containing lipid vacuoles were seen in $4{\cdot}6 \pm 1{\cdot}8$% of glomeruli on day 3. There was a progressive increase in the number and size of foam cells, and by week 6 virtually all glomeruli ($97{\cdot}6 \pm 1{\cdot}6$%) contained foam cells, and segmental clusters of foam cells associated with capsular adhesions and destruction of the glomerular tuft were seen in $5{\cdot}1 \pm 1{\cdot}0$% of glomeruli.

Glomerular M-CSF expression and macrophage accumulation

In situ hybridization showed that a small number of resident glomerular cells, probably podocytes, constitutively expressed M-CSF mRNA in ExHC rats fed on a normal diet (Fig. 1a). In addition, constitutive M-CSF mRNA expression was seen in some thick ascending limbs of Henle, distal and medullary tubules.

Coincident with the onset of hypercholesterolaemia on day 3, there was a significant increase in the number of glomerular M-CSF$^+$ cells, and this increased further over the 6-week period of the HCD (Figs 1b and 2a). Double-labelling showed that some infiltrating macrophages expressed M-CSF mRNA, but most M-CSF$^+$ cells were intrinsic glomerular cells (Figs 1b,c and 2a). Mesangial cells and podocytes appeared to be the main glomerular cell type expressing M-CSF mRNA on the HCD. Importantly, up-regulation of glomerular M-CSF expression preceded glomerular macrophage accumulation (Figs 1b and 2a), and there was a highly significant correlation between the number of glomerular M-CSF$^+$ cells and accumulation of ED1$^+$ macrophages over the 6-week period on the HCD (Fig. 2a,b). Some ED1$^+$ macrophage-derived foam cells showed M-CSF mRNA expression.

Glomerular MIF expression and macrophage accumulation

Consistent with a previous study of Sprague-Dawley rats [18], *in situ* hybridization demonstrated constitutive MIF mRNA expression by a small number of resident glomerular cells in ExHC rats, which were most probably podocytes (Fig. 1d). Within 3 days of the induction of the HCD there was a dramatic increase in glomerular MIF mRNA expression (Fig. 1e), which increased further over the 6-week period (Figs 1f and 3a). Although double-staining identified MIF expression by 32% of ED1$^+$ macrophages, including some foam cells, the main source of the increased glomerular MIF production was intrinsic glomerular cells—particularly podocytes (Fig. 1f). A similar pattern of glomerular MIF expression was also seen by staining with the anti-MIF antibody (data not shown). The up-regulation of glomerular MIF expression preceded monocyte infiltration (Figs 1e and 3a), and there was an excellent correlation between glomerular MIF expression and macrophage accumulation throughout the period on the HCD (Fig. 3b).

Glomerular macrophage proliferation

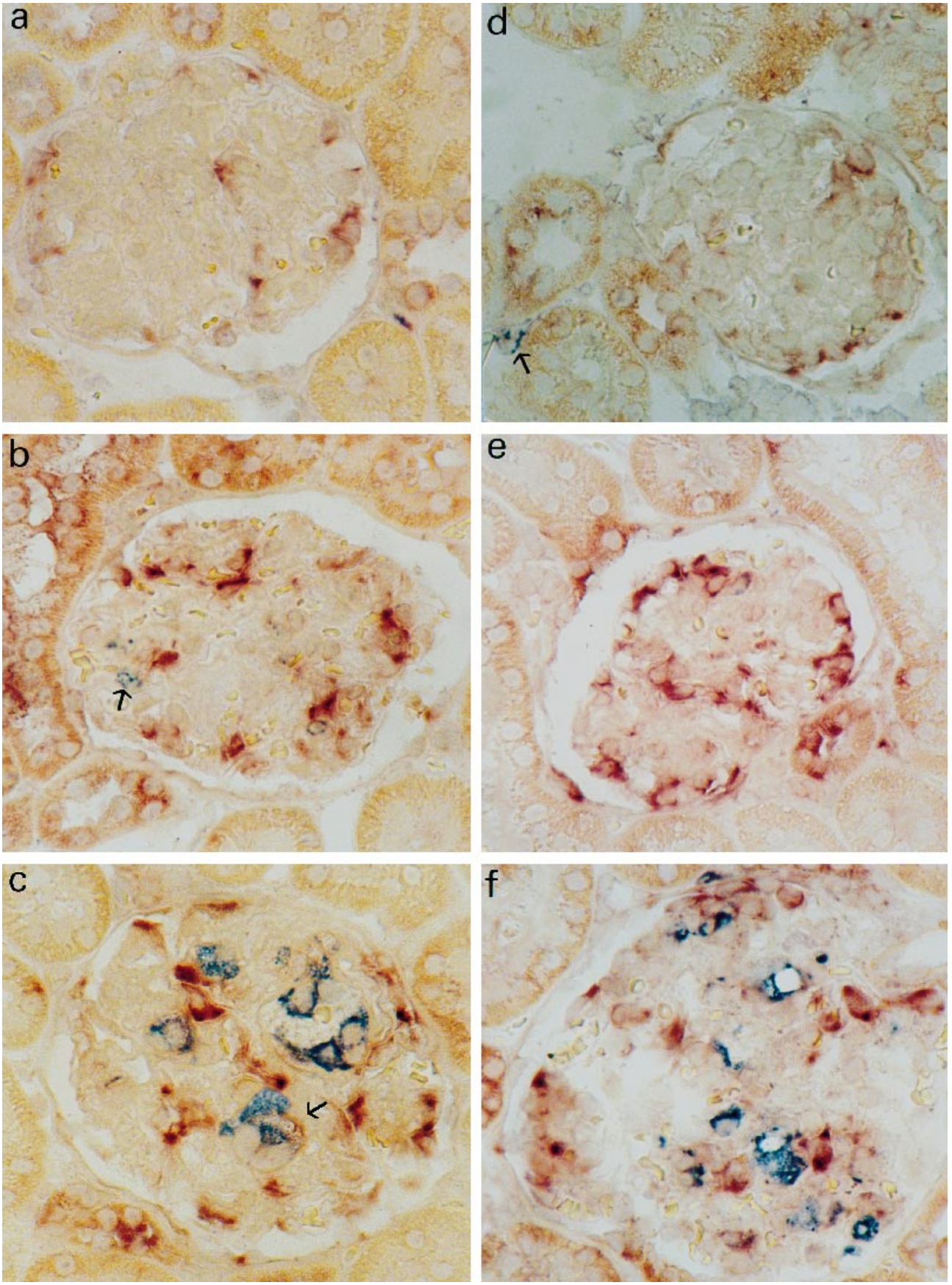
Occasional proliferating glomerular cells were labelled with the PC-10 (anti-PCNA) MoAb in ExHC rats fed a normal diet. There was no increase in the number of PCNA$^+$ cells over the 6-week period on the HCD, and only rare ED1$^+$ PCNA$^+$ proliferating macrophages were seen (data not shown).

DISCUSSION

This study has shown that hypercholesterolaemia induces up-regulation of M-CSF and MIF expression by intrinsic glomerular cells, which may be an important mechanism of monocyte recruitment and accumulation during the development of lipid-induced renal injury. This postulate is based upon three findings. First, up-regulation of glomerular M-CSF and MIF expression was coincident with the induction of hypercholesterolaemia. Second, up-regulation of M-CSF and MIF expression preceded monocyte recruitment. Third, there was an excellent correlation between up-regulation of M-CSF and MIF expression and glomerular macrophage accumulation over the 6-week period of the HCD.

Several lines of evidence support a role for M-CSF in monocyte recruitment in the development of lipid-induced renal injury. M-CSF is a potent chemotactic molecule for monocytes, and low-density lipoprotein (LDL) has been shown to induce M-CSF synthesis and secretion by cultured mesangial cells and aortic endothelial cells [27]. Up-regulation of M-CSF expression by LDL operates through activation of the nuclear transcription factor NF-κB [28], a factor which induces the expression of a number of molecules involved in the inflammatory response, such as leucocyte adhesion molecules, which are also rapidly up-regulated in ExHC rats fed an HCD [22]. Local production of M-CSF has been described in vascular atherosclerotic lesions [9–11], and M-CSF may contribute to foam cell formation by up-regulating monocyte expression of the LDL receptor and lipid metabolism [29–31]. In addition, genetic studies in mice have demonstrated a key role for M-CSF and its effects upon macrophage development and function in the pathogenesis of atherosclerosis [32].

This is the first study to identify MIF expression within lipid-induced lesions. Currently, nothing is known of whether hypercholesterolaemia up-regulates MIF expression through a direct or



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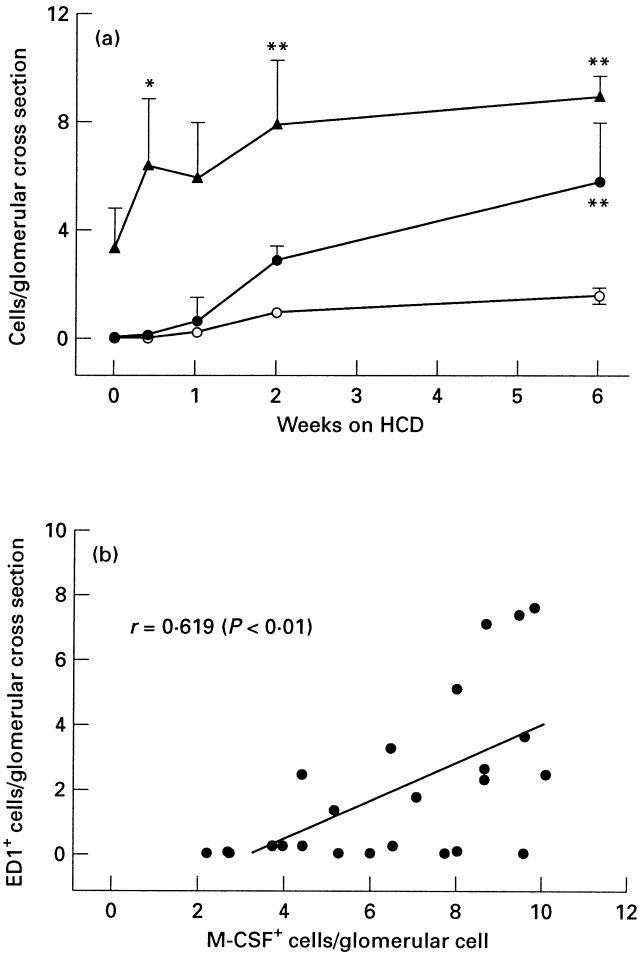

Fig. 2. Quantification of glomerular M-CSF mRNA expression and ED1$^+$ macrophage infiltration in ExHC rats fed on a high cholesterol diet (HCD). (a) Total number of M-CSF mRNA$^+$ cells (▲), total ED1$^+$ cells (●) and M-CSF$^+$ ED1$^+$ cells (○) were scored in 20 glomeruli from each animal. Data are the mean ± s.d. for groups of five animals. *$P < 0{\cdot}05$; **$P < 0{\cdot}001$ *versus* day 0 by ANOVA. (b) Pearson single correlation analysis of glomerular M-CSF$^+$ cells and ED1$^+$ cells for all animals on days 0, 3 and weeks 1, 2 and 6 of the HCD.

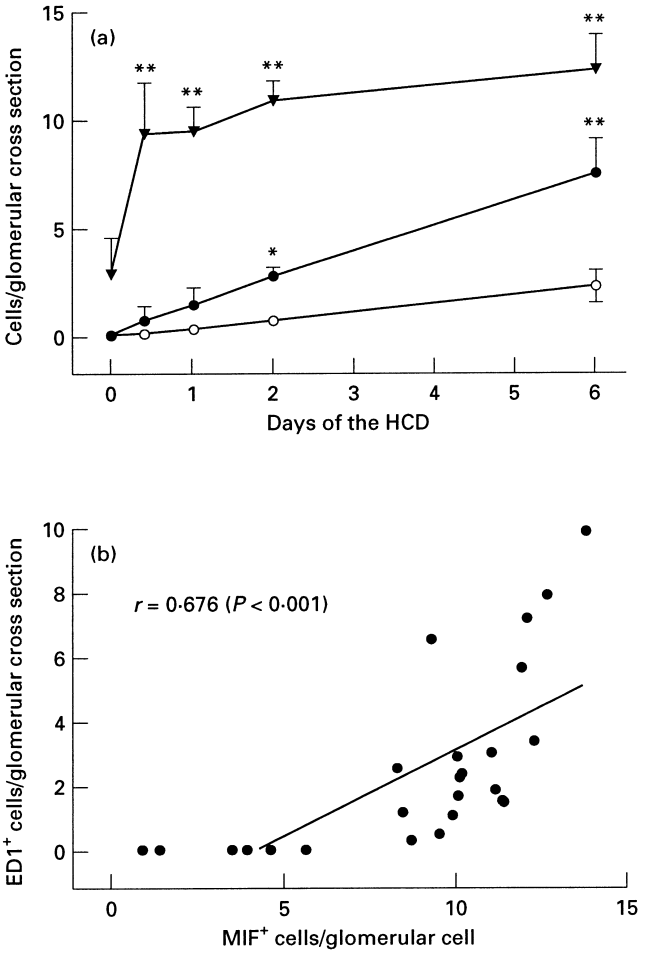

Fig. 3. Quantification of glomerular MIF mRNA expression and ED1$^+$ macrophage infiltration in ExHC rats fed on a high cholesterol diet (HCD). (a) Total number of MIF mRNA$^+$ cells (▼), total ED1$^+$ cells (●) and MIF$^+$ ED1$^+$ cells (○) were scored in 20 glomeruli from each animal. Data are the mean ± s.d. for groups of five animals. *$P < 0{\cdot}05$; **$P < 0{\cdot}001$ *versus* day 0 by ANOVA. (b) Pearson single correlation analysis of glomerular MIF$^+$ cells and ED1$^+$ cells for all animals on days 0, 3 and weeks 1, 2 and 6 of the HCD.

indirect pathway. The ability of MIF to activate macrophages and inhibit their migration plays a key role in macrophage accumulation and tissue damage in animal models of DTH and crescentic glomerulonephritis [16,18]. Thus, MIF may play a similar key role in promoting macrophage accumulation and the subsequent development of atherosclerotic lesions at sites of lipid deposition.

Another possible mechanism of macrophage accumulation in lipid-induced glomerular injury is local macrophage proliferation. This has been shown to be a major mechanism of macrophage accumulation in models of severe tissue injury, but is much less prominent where tissue injury is mild (reviewed in [33]). Low levels of local macrophage proliferation together with M-CSF expression have been reported in atherosclerotic lesions in an HCD model in the rabbit [10]. However, despite the presence of

Fig. 1. Up-regulation of M-CSF and MIF mRNA expression and glomerular macrophage accumulation in ExHC rats. *In situ* hybridization of M-CSF (a–c) and MIF (d–f) mRNA was combined with ED1 immunostaining (a–f). (a) Constitutive M-CSF mRNA expression (brown/purple) by resident glomerular cells, probably podocytes, on day 0. (b) Up-regulation of M-CSF mRNA expression (brown/purple) by some mesangial-like cells on day 3 of the high cholesterol diet (HCD), with only one ED1$^+$ (blue) macrophage present (arrow). (c) Strong M-CSF mRNA expression by resident glomerular cells and M-CSF expression by one of the ED1$^+$ (blue) macrophage-derived foam cells (arrow) on week 6 of the HCD. (d) Constitutive MIF mRNA expression (brown/purple) by resident glomerular cells on day 0, probably podocytes, with an interstitial ED1$^+$ (blue) macrophage also seen (arrow). (e) Marked up-regulation of glomerular MIF expression by resident glomerular cells, presumably podocytes and mesangial cells, on day 3 of the HCD prior to monocyte recruitment. (f) Many intrinsic glomerular cells express MIF mRNA on week 6 of the HCD, while only some ED1$^+$ (blue) macrophages and foam cells express MIF mRNA. (Original mag. ×400.)

up-regulated M-CSF expression in our model of lipid-induced glomerular injury in ExHC rats, we found no evidence of local macrophage proliferation during lesion formation. This apparent difference could relate to the stage of lesion formation examined in the two studies, or reflect an intrinsic difference in the development of glomerular and arterial lesions.

In conclusion, this study has shown that hypercholesterolaemia in susceptible ExHC rats rapidly induces M-CSF and MIF expression by intrinsic glomerular cells which, in turn, is associated with glomerular monocyte recruitment and the development of lipid-induced glomerular injury. This mechanism may be of general importance in the recruitment of blood monocytes into lipid-induced lesions.

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