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Expression of lymphotactin mRNA in experimental crescentic glomerulonephritis

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SUMMARY

Lymphotactin (LTN) is the sole member of C chemokine, the third subfamily of chemokines. LTN has been shown to be a chemoattractant specific for $CD8^+$ cells and/or natural killer (NK) cells, and to be produced by $CD8^+$ T cells, NK cells, and mast cells. However, there have been no reports describing its expression in clinical or experimental models of diseases so far. Since glomerular infiltration of $CD8^+$ cells is prominent in an animal model of crescentic glomerulonephritis induced in WKY rats by an injection of anti-glomerular basement membrane antibody, we investigated the gene expression of LTN in this model. LTN mRNA was not detected in normal glomeruli but was detected at 0·5 h after the antibody injection, which detection preceded the infiltration of $CD8^+$ cells. The expression of LTN mRNA peaked on day 3 and decreased thereafter. We next studied the expression of LTN mRNA in cultured glomerular and vascular cells, and found that glomerular mesangial and vascular endothelial cells could express LTN mRNA when stimulated with IL-1β. These results indicate that the gene expression of LTN is enhanced in the animal model of glomerulonephritis and that intrinsic renal cells are the potential source of the gene expression of LTN in the kidney.

Keywords chemokine crescentic glomerulonephritis lymphotactin

INTRODUCTION

The chemokines are a large family of chemoattractant cytokines whose members have been divided into three groups, CXC, CC and C subfamilies, on the basis of the positions of four invariant cysteines [1–3]. The first two cysteines of the CXC chemokines are separated by a single amino acid residue, while the CC chemokines all have the first two cysteines immediately adjacent to one another. Lymphotactin (LTN), the sole member of the C chemokine subfamily, lacks two of the four characteristic cysteine residues and thus can form only one of the two intra-chain disulphide bonds found in the CXC and CC chemokines [4]. Although LTN has amino acid similarities with the CC chemokines, it also has an extended carboxyl terminus that is unusual among chemokines and is essential for its activity [5]. LTN has been shown to be active on T lymphocytes, especially on $CD8^+$ cells, but not on neutrophils or monocytes [4,6]. LTN mRNA is mainly expressed by activated $CD8^+$ T cells [4,5,7], and also by natural killer (NK) cells [5], $\gamma\delta$ T cells [8], and mast cells [9]. Although the production of LTN is induced in these cells *in vitro*, there have been no reports showing that LTN is induced in any types of human diseases or experimental models of diseases so far.

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Many studies suggest the potential role of CXC and CC chemokines in recruitment and activation of leucocytes in various diseases, including renal diseases [10,11]. In crescentic glomerulonephritis, a most severe form of glomerular disease, glomerular infiltration of both monocytes and lymphocytes is observed in clinical cases as well as in experimental animal models [12], and our previous studies [13,14] have demonstrated that the glomerular expression of mRNAs for MCP-1 and five other CC chemokines is enhanced in a rat model of the disease. In this model, which is induced in WKY rats by an injection of a small dose of anti-glomerular basement membrane (GBM) antibody, the enhanced gene expression of the chemokines was seen in an early phase of the disease, in parallel with an increase in the number of glomerular monocytes/macrophages. The early infiltration of $CD8^+$ cells concomitant with that of monocytes/macrophages into the glomeruli is a distinctive feature of this model, and the importance of $CD8^+$ cells in the initiation and/or progression of the disease has been suggested [15].

The specific action of LTN towards $CD8^+$ cells prompted us to examine whether LTN mRNA is expressed in glomeruli of rats with crescentic glomerulonephritis. Here we show an up-regulation of the expression of LTN mRNA that precedes the increase of $CD8^+$ cells in glomeruli. Furthermore, we show that cultured glomerular cells could express LTN mRNA.

MATERIALS AND METHODS

Animal model

Crescentic glomerulonephritis was induced in WKY rats (6 weeks old; Charles River Japan, Atsugi, Japan) by an i.v. injection of anti-GBM antiserum (20 μl/100 g body weight) as described previously [13]. Animals were killed 30 min, 4 h, 24 h, 3 days and 7 days after the injection of the antiserum (five rats for each time point). Five rats were killed at time 0 without injection of antiserum. The kidneys were perfused from the abdominal aorta with ice-cold sterile PBS. Half of the right kidney of each animal was used for histological studies, and the remaining one and a half kidneys obtained from each of five rats in each group were pooled and used for preparation of glomeruli. Glomeruli were also isolated from control rats that had been injected 24 h or 7 days previously with normal rabbit serum (five rats for each time point). For sampling urine, rats were maintained in metabolic cages for collection of urine and the urinary excretion of protein was monitored by the biuret method.

Histology

Histological analyses were performed as detailed previously [13]. Briefly, tissues for light microscopy were fixed in paraformaldehyde, embedded in polyester wax, sectioned, and stained with haematoxylin and eosin. Tissues for immunoperoxidase staining were fixed in 2% periodate-lysine-paraformaldehyde, embedded in OCT compound, and sectioned. Sections were incubated with mouse anti-rat MoAbs including ED-1 (reactive with monocytes/macrophages) or OX8 (with $CD8^+$ cells) at 4°C overnight. The sections were then incubated with biotinylated horse anti-mouse IgG and stained with an ABC staining kit (Vector Labs, Burlingame, CA). Positive cells in 20 glomeruli per section obtained from each animal were counted in blind fashion.

Cell culture

Rat endothelial cells were obtained from the thoracic aorta, as described previously [16]. Rat glomerular mesangial cells were obtained from primary glomerular explants of Sprague-Dawley rats [16]. Rat glomerular epithelial cells were cultured as described [17].

Northern blot analysis

Since cDNA for rat LTN mRNA had not been cloned, it was obtained as follows. Primers were designed based on the sequences of murine LTN [4]; the forward primers were 5′-AGGAAGACCT-CAGCCATGAG-3′ and the reverse primer was 5′-GAGGCTGT-TACCCAGTCAGGGT-3′. In addition, a HindIII or EcoRI restriction enzyme site was incorporated into the 5′ end of each primer. The reverse transcription-polymerase chain reaction (RT-PCR) was performed with RNA extracted from glomeruli of rats that had been injected with anti-GBM antibody 24 h before, as described above. The sequence of the cDNA was determined with an automated DNA sequencer (ABI PRISM 377; Perkin Elmer, Foster City, CA) and was found to have 88·9% homology with that of murine LTN.

Total RNA was prepared from glomeruli by the AGPC method [18], fractionated by electrophoresis on a 1·2% agarose gel, and transferred to a nitrocellulose membrane. The membrane was baked at 80°C for 2 h and used for hybridization with ^{32}P-cDNA for rat LTN or with ^{32}P-cDNA for rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA [19]. The hybridized membrane was washed three times with 2×SSC containing 0·1% SDS at room temperature for 5 min each and then with 0·2×SSC containing 0·1% SDS at 50°C for 20 min. Imaging of radioactive signals was performed with an Imaging Analyser (BAS1000Mac; Fuji Photo Film Co., Tokyo, Japan). PSL values for LTN mRNA were normalized to those for GAPDH mRNA.

RT-PCR

Two micrograms of total RNA were reverse transcribed by use of the SuperScript II Preamplification System (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Subsequently, PCR for LTN was performed in a 50-μl reaction volume including 1·25 U *Taq* DNA polymerase (Promega, Madison, WI), 0·1 μM concentration of each primer, and 10 μl of cDNA template. Primers for experiments using RNA of glomeruli or RAW 264 cells, which were designed based on the sequence of murine LTN [4], were 5′-GCGAATTCAGCAAGACCTCAGC-CATGAG-3′ (5′ primer) and 5′-CCAAGCTTGAGGCTGTTACC-CAGTCAGGGT-3′ (3′ primer), defining a fragment of 383 bp. The amplification reaction was carried out for a total of 33 cycles as follows: 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. Rat cultured cells contained a lower amount of LTN mRNA, and the RT-PCR system described above was not sensitive enough for them. Therefore, primers were designed based on the rat cDNA sequences, 5′-CCTGGGAGTCTGCTGCTTCG-3′ (5′ primer) and 5′-TGGCGGACCTCTGGGCTTGT-3′ (3′ primer), defining a fragment of 313 bp, and were used for experiments using RNA of rat cultured cells. The amplification reaction was carried out for a total of 33 cycles (glomerular mesangial cells) or 40 cycles (endothelial cells) as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. PCR for GAPDH was performed as described [14]. The products of PCR were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Statistical analysis

All values were expressed as the means ± s.d. Data were analysed by ANOVA followed by Fisher's PLSD procedure or by Mann–Whitney *U*-test, and a value of $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

An injection of anti-GBM antibody into WKY rats induced crescentic glomerulonephritis, as described previously [13]. On day 3, abnormal urinary protein excretion appeared (a-GBM rats, 99 ± 39 mg/24 h; control rats, 2·4 ± 1·5 mg/24 h; $P < 0{\cdot}01$) and crescentic glomeruli became prominent. An accumulation of $CD8^+$ cells in glomeruli was found 4 h after injection of the antiserum; the cell number peaked at 24 h and decreased thereafter (Fig. 1a). Infiltration of monocytes/macrophages was observed at 0·5 h, became statistically significant at 4 h and reached its maximum level on day 3 (Fig. 1b).

The expression of LTN mRNA was not observed in normal glomeruli, became detectable at 0·5 h, and seemed to increase continuously until day 7 by RT-PCR (Fig. 2a). For quantitative analysis, we next examined the expression of LTN mRNA by Northern blot analysis. Figure 2b,c shows that the expression of LTN mRNA was detected at 4 h, peaked on day 3 and then decreased on day 7. The size of transcripts was about 0·8 kb,

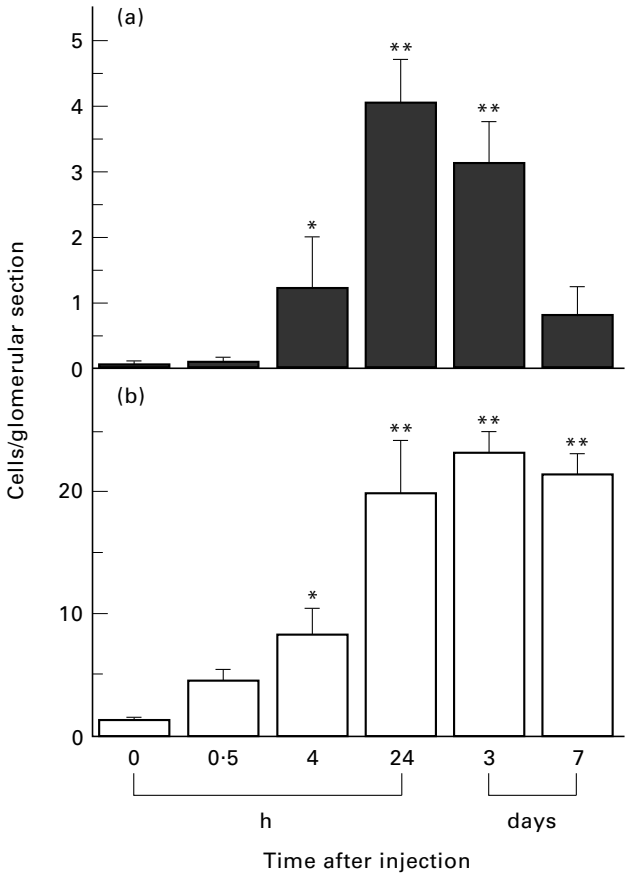

Fig. 1. Time course of the infiltration into glomeruli of $CD8^+$ cells (a) and monocytes/macrophages (b). $*P < 0{\cdot}01$; $**P < 0{\cdot}0001$ compared with 0 h.

which is similar to that of human LTN transcripts [20]. Glomerular RNA from control rats injected with normal rabbit serum showed no expression of LTN mRNA by Northern blot analysis and very low levels of expression by RT-PCR (data not shown).

Finally we investigated whether LTN mRNA could be expressed in cultured cells. Figure 3a shows that glomerular mesangial cells expressed LTN mRNA when they were stimulated with IL-1β. A time-dependent increase in the expression of LTN mRNA was also observed in activated vascular endothelial cells (Fig. 3b). IL-1β-stimulated glomerular epithelial cells or lipopolysaccharide (LPS)-stimulated cells of the macrophage cell line RAW 264 showed no expression of LTN mRNA under the conditions where the expression of CC chemokines was up-regulated (data not shown).

DISCUSSION

This study has demonstrated that the expression of LTN mRNA is enhanced in glomeruli in an experimental model of crescentic glomerulonephritis. To our knowledge, this is the first report describing the enhanced gene expression of LTN in disease state.

The expression of LTN mRNA was detected by a sensitive RT-PCR method at 0·5 h, which detection preceded the increase in the number of $CD8^+$ cells, the possible target cells of LTN, in glomeruli: the number of $CD8^+$ cells remained normal at 0·5 h and increased at 4 h. Although the production of LTN protein has not yet been confirmed, it seems conceivable that LTN induced in glomeruli may contribute to the infiltration of $CD8^+$ cells.

It is reported that LTN mRNA is expressed in $CD8^+$ T cells but not in $CD4^+$ T cells or in monocytes [7]. The present study confirmed that a murine macrophage cell line RAW 264 does not express LTN mRNA. Therefore, the glomerular expression of LTN mRNA at 0·5 h could not be attributed to infiltrating monocytes/macrophages. One possible explanation for the mRNA expression is that resident glomerular $CD8^+$ cells were activated after the binding of antibody to GBM and began to express LTN mRNA, although the number of $CD8^+$ cells in glomeruli was very low. Another possibility is that glomerular intrinsic cells were responsible for the expression of the mRNA. As shown in this study, mesangial and vascular endothelial cells in culture expressed LTN mRNA, when activated by IL-1β. Therefore, it is possible that mesangial and/or glomerular endothelial cells are responsible for the early expression of LTN mRNA in this model. These cells are the first examples of cells other than haematopoietic cells to express LTN mRNA.

Although several early studies on LTN showed that it is a selective chemoattractant for lymphocytes, especially $CD8^+$ T cells [4,6], the activity of LTN is still controversial [3]. Recently it was suggested that LTN also shows chemotactic activities toward NK cells [5,21]. On the other hand, Dorner *et al*. [22] argued that LTN purified from natural sources displayed

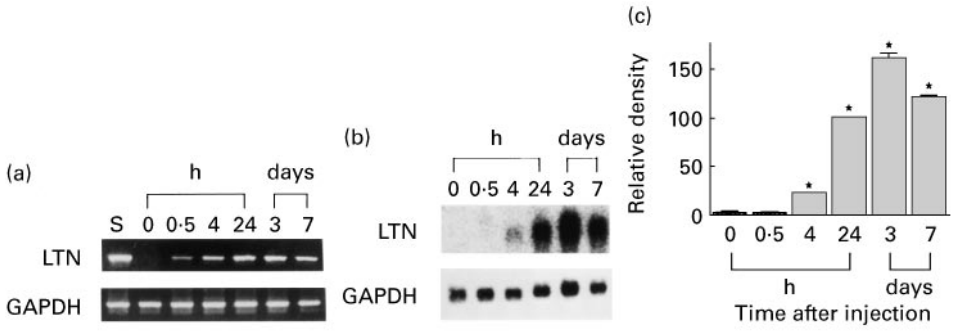

Fig. 2. Time course of the glomerular expression of lymphotactin (LTN) mRNA after a single injection of anti-glomerular basement membrane (GBM) antibody. (a) Reverse transcription-polymerase chain reaction (RT-PCR). Lane S represents total RNA from concanavalin A-stimulated rat splenocytes. (b) Northern blot analysis. Results representative of those of three experiments are shown. (c) Relative density of LTN mRNA in Northern blot analysis. The levels were normalized to those of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA and are expressed relative to the level for glomeruli at 24 h (100%). Means ± s.d. of three experiments are shown. $*P < 0{\cdot}0001$ compared with 0 h.

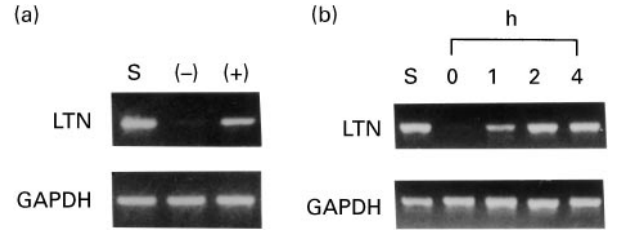

Fig. 3. Expression of lymphotactin (LTN) mRNA in cultured cells. (a) Mesangial cells. They were incubated without (–) or with (+) IL-1β (5 ng/ml) for 4 h. (b) Aortic endothelial cells. Time course of the expression of LTN mRNA in cells stimulated with IL-1β (5 ng/ml) for this time period is given. Lane S represents total RNA from concanavalin A-stimulated rat splenocytes.

chemokinesis activity toward $CD4^+$ and $CD8^+$ T cells, but no chemotactic activity toward them. They posed the question whether LTN should be functionally grouped in the family of chemokines. Therefore, the glomerular expression of LTN mRNA shown in this study might not contribute to the infiltration of $CD8^+$ cells, but have some unknown function in the mechanism of disease progression. We believe that inhibition studies on this model by neutralizing antibody or antagonists to LTN would be useful to elucidate the function of LTN *in vivo*. Since the infiltrating $CD8^+$ cells in this model are suggested to be NK cells rather than T lymphocytes [23], the definitive identification of these infiltrating cells would be helpful for understanding the role of LTN.

Leucocytes are considered to play a crucial role in the crescent formation in crescentic glomerulonephritis [24]. Cellular crescent, an early stage of crescent formation, is formed by proliferation of intrinsic glomerular epithelial cells and by infiltration of leucocytes [12]. Observations of animal models and clinical cases of the disease suggest that the cellular crescent can spontaneously resolve if there is no continued leucocyte accumulation [12]. Since $CD8^+$ T cells and/or NK cells in addition to macrophages are thought to be involved in the pathogenesis of crescentic glomerulonephritis in humans [25–27] as well as in an animal model [23], the identification of the chemoattractant(s) responsible for the infiltration of these cells would be helpful to define the target molecules for treatment of crescentic glomerulonephritis.

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