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The Novel Regulatory Mechanism of Fas System-Mediated Apoptosis in Mesangial Cells: Implication to Mesangial Proliferative Glomerulonephritis

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Fas Ag is a cell surface molecule that transduces the signal for apoptosis. Since mesangial cells (MC) play important roles in regulating glomerulonephritis, we investigated regulatory mechanisms of Fas system in MC. Fas Ag was expressed on MC from normal mice. This Fas Ag expression was down-regulated by inducing proliferation with platelet-derived growth factor or 18% fetal bovine serum, but was reversed when cycloheximide was added to the culture. Anti-Fas Ab alone did not induce apoptosis in MC, but MC became susceptible to apoptosis induced by anti-Fas Ab if actinomycin D or cycloheximide was added. Noteworthy findings are that mRNA of Fas ligand was expressed in MC. Taken together, MC appears to control the proliferation by regulating Fas system-mediated apoptosis, at least in part, through an autoregulatory mechanism with Fas Ag and Fas ligand expressed on their own MC.

Fas Ag is a cell surface molecule which transduces the apoptotic signal into cells. This molecule is expressed not only on lymphocytes but also on a variety of cells including hepatocytes and mesangial cells (MC) (1). MC reside in glomeruli and regulate renal homeostasis (2). Deposition of immune complexes in the mesangial area stimulates the proliferation of MC, leading to mesangial proliferative glomerulonephritis (MesGN) as seen in lupus nephritis and IgA nephropathy. MesGN has been extensively investigated in the lpr (lymphoproliferation) mouse, model of systemic lupus erythematosus (3). These mice generate various autoantibodies including anti-DNA and anti-Sm antibodies, which cause immune complex nephritis (3). Furthermore, Liu et al. showed that platelet-derived growth factor (PDGF) elicits a more pronounced proliferation in the MC of lpr mice than in those of normal mice (4). In contrast, MC of lpr mice are relatively insensitive to the suppressive effects of transforming growth factor-β (TGF-β) (4).

The lpr mutation is the insertion of an early transposable element to intron 2 of the Fas Ag gene, which severely hampers Fas Ag expression (5). These findings suggest that a Fas defect on MC contributes to the aggravation of MesGN. From this perspective, we clarified the regulatory mechanism of the Fas system in MC using in vitro system.

MATERIAL AND METHODS

Mice. MRL/MpJ-+/+ and MRL/MpJ/-lpr/lpr mice were purchased from Japan SLC. Inc. (Hamamatsu, Japan) and bred in a specific pathogen free environment.

MC isolation and growth. The glomeruli from a 5-week-old mouse isolated by a sieving as previously reported (4).

Assay for MC proliferation. MC (8×10^3) were stimulated with human-PDGF-BB (GIBCO BRL, MD, USA), human TGF-β_1 (provided by Kirin Corp.) or 18% FBS for 72 hours. The cells were incubated with 1μCi/ml of [^{3}H] thymidine and cultured for another 24 hours. Thereafter the medium was aspirated. The cells were dispersed in 50 μl of 0.25% trypsin with 0.025% EDTA per well, harvested and the amount of incorporated radioactivity was measured using a liquid scintillation counter.

Assay for apoptosis of MC. MC (4×10^4) was cultured with anti-Fas Ab (Jo2) in the absence or presence of actinomycin

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D (Sigma Chemical Co., Deisenhofen, Germany) or cycloheximide (Wako Pure Chemical Industries Ltd., Japan) was added to the cultures. Viable MC were counted by trypan blue staining 20 hours later.

Flow cytometry. MC (4×10^4) were stained with anti-mouse Fas Ag mAb (10 μg/ml) (clone: Jo2) and with FITC-conjugated anti-hamster IgG (Caltag, CA, USA) (10 μg/ml). After the final wash, cells were resuspended in 0.3 ml buffers containing 10 ng/ml propidium iodide. Control samples were stained with second Ab alone. Flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA) and data collected on viable cells were analyzed using Consort 30 software.

DNA extraction. MC were harvested and pelleted. The cells were resuspended in lysis buffer containing RNase A (20 μg/ml). After incubating for 24 hours at 37°C, DNA was extracted with phenol/chloroform, precipitated with ethanol and resuspended in TE buffer. DNA (5 μg) was resolved by electrophoresis in 1% agarose.

Northern blots. Total RNA was extracted using guanidinium isothiocyanate (6) and resolved by electrophoresis in 1% agarose gels containing 1.8% formaldehyde. RNA was transferred to nitrocellulose membranes (BA85, Schleicher & Schuell Inc., Dassel, Germany). Membranes were prehybridized, hybridized with different DNA probes (5,7) labeled with ^{32}P by random priming. The membranes were washed at high stringency, then exposed to X-ray films at −70 °C with intensifying screens. Autoradiograms were quantified by means of densitometric scanning using a FUJIX Bio-Image Analyzer BAS 2000 (Fuji Photofilm Co., Tokyo, Japan).

mRNA quantitation by PCR. Total RNA was extracted from cultured MC using guanidinium isothiocyanate method as described (6) and cDNA was synthesized with reverse transcriptase (Takara, Japan). The cDNA was transferred to a tube containing dNTPs, Taq polymerase (Takara, Japan), random primer mixtures, and 1.4 mM $MgCl_2$. The amplification conditions were 95 °C for 5 min. followed by 35 cycles at 95 °C for 30 sec., 60 °C for 30 sec. and 72 °C for 1 min. using mouse Fas ligand (8) and β-actin primers (7). After amplification, 10 μl of the PCR products were separated on 1.6% agarose gels and stained by ethidium bromide.

RESULTS

Fas Ag expression on resting MC. MC from MRL-+/+ and MRL-lpr/lpr mice grew to subconfluence in the presence of 18% FBS, but became quiescent when cultured in medium containing 0.5% FBS for 48 hours. Single cell suspensions were stained with anti-mouse Fas Ab and then with FITC-conjugated anti-hamster IgG. MC from MRL-+/+ mice expressed Fas Ag (Fig. 1A), whereas those from MRL-lpr/lpr mice did not (Fig. 1A), as evaluated by flow cytometric analysis.

The relationship between the proliferative state and the expression ability of Fas Ag in MC. We evaluated the influence of the culture condition on the proliferative state. Resting MC cultured in medium containing 0.5% FBS were stimulated with PDGF (20 ng/ml), 18% FBS, or 18% FBS plus TGF-β (30 ng/ml) for 72 hours. Both PDGF and 18% FBS induced proliferative response in MC from MRL-+/+ mice, although the latter agent proliferated MC about twice as much as the former (Table 1). By contrast, TGF-β rather suppressed the proliferative activity induced by 18% FBS, but it was still higher than that in 0.5% FBS (Table 1).

We examined the effects of culture conditions on the expression of Fas Ag. PDGF and 18% FBS down-regulated the expression of Fas Ag on MC compared with that cultured in 0.5% FBS (Fig.

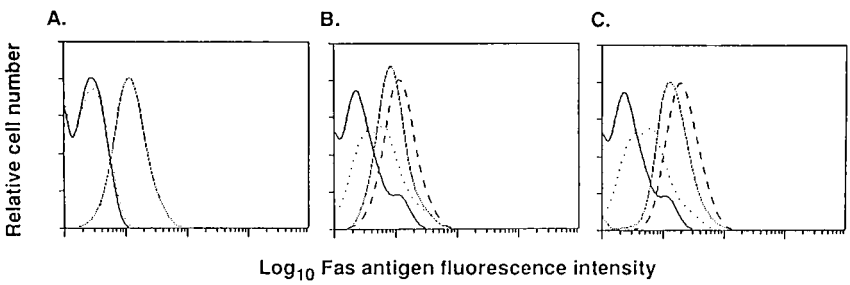

FIG. 1. Effect of cytokines on Fas antigen expression on MC. (A) MC from MRL-+/+ (--------) and MRL -lpr/lpr (- - - -) mice were cultured in medium containing 0.5% FBS for 48 hours. (B) MC from MRL-+/+ mice in medium containing 0.5% FBS (– –) were stimulated with PDGF (20 ng/ml) (-------) or 18% FBS (- - - -) for 72 hours. (C) MC from MRL-+/+ mice were cultured in medium containing 0.5% FBS (– –), 18% FBS (- - - -), or 18% FBS and TGF-β (30 ng/ml) (--------) for 72 hours. A single cell suspension of MC was stained with anti-Fas Ab followed by FITC-conjugated hamster IgG and analyzed by flow cytometry. Background staining (—) represents labeling with the second antibody alone.

TABLE 1
Proliferative Responses of MC

Stimulation	^{3}H-Thymidine incorporation (cpm × 10^2)
0.5% FBS	2.59 ± 0.5
PDGF (20 ng/ml)	6.36 ± 0.8
18% FBS	12.6 ± 0.9
18% FBS and TGF-β (30 ng/ml)	7.52 ± 1.5

MC from MRL-+/+ mice were cultured in media containing 0.5% FBS, 0.5% FBS and PDGF (20 ng/ml), 18% FBS or 18% FBS and TGF-β (30 ng/ml) for 3 days. The final 24 hour ^{3}H-Thymidine uptake was assayed. Results represent means ± SD of triplicate assays from three independent experiments.

1B). On the other hand, the intensity of Fas Ag expression on MC in 18 % FBS plus TGF-β increased compared with that in 18% FBS but it was not lower than that in 0.5% FBS (Fig. 1C).

Cycloheximide reverses the down-regulation of Fas Ag expression. To assess whether new protein synthesis is required for the down-regulation of Fas Ag, MC were cultured in the presence of cycloheximide for 6 hours and mRNA for Fas Ag was assessed by Northern blotting. The level of mRNA was relatively estimated as the expression ratio of Fas Ag to β-actin. The relative mRNA

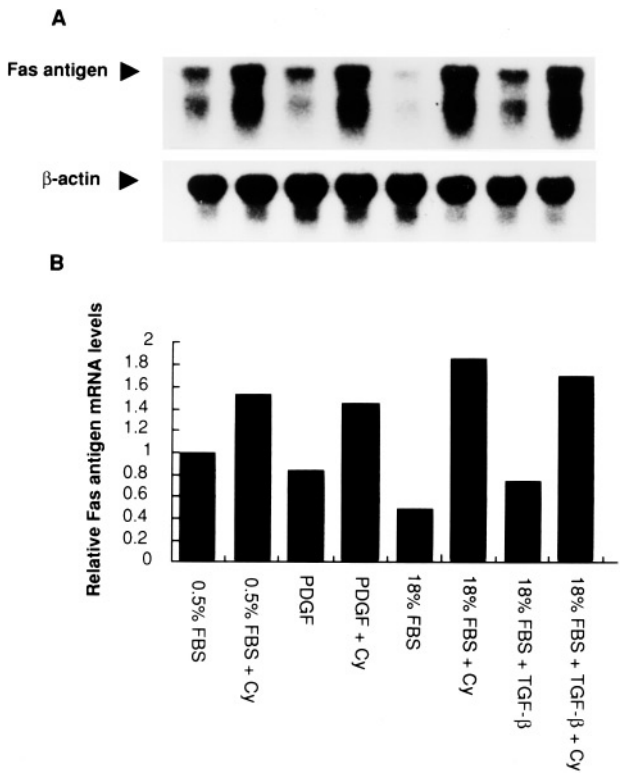

FIG. 2. Effect of cycloheximide on the down-regulation of Fas antigen. MC from MRL-+/+ mice were stimulated with PDGF (20 ng/ml), PDGF plus cycloheximide (50 mg/ml), 18% FBS, 18% FBS plus cycloheximide (50 mg/ml), 18% FBS plus TGF-β (30 ng/ml), or 18% FBS plus TGF-β (30 ng/ml) plus cycloheximide (50 mg/ml) for 6 hours. Total RNA was extracted and assessed by (A) Northern blotting with mouse Fas antigen or β-actin probe. (B) The relative Fas antigen mRNA levels were expressed as Fas antigen mRNA/β-actin mRNA from each sample/that from MC cultured in 0.5% FBS.

level of Fas Ag decreased in MC cultured with 0.5% FBS plus PDGF, or with 18% FBS compared with those cultured with 0.5% FBS (Fig. 2A, B), which correlated with their surface expression as evaluated by FACS analysis. Next, MC cultured with 0.5% FBS were incubated with cycloheximide, PDGF plus cycloheximide, or 18% FBS plus cycloheximide for 6 hours, and the total RNA of each group was assessed by Northern blotting. The down-regulated expression of Fas Ag according to stimulation with PDGF or 18% FBS (Fig. 2A, B) was reversed by the co-culture with cycloheximide. Moreover, the mRNA level of Fas Ag of MC cultured with cycloheximide plus PDGF or 18% FBS was higher than that of MC cultured with 0.5% FBS, and almost equal to that cultured in 0.5% FBS plus cycloheximide (Fig 2 A, B). Cycloheximide did not affect the expression levels of β-actin mRNA (Fig. 2A). These findings showed that new protein synthesis is required for this down-regulation.

Apoptosis of MC with anti-Fas Ab. To investigate whether cultured MC from MRL-+/+ mouse undergo apoptosis through Fas Ag, MC cultured with 0.5% FBS were incubated with anti-Fas Ab for 20 hours and viable cells were counted by trypan blue staining. The incubation with anti-Fas Ab alone did not induce apoptosis in MC even at a concentration of 1.0 μg/ml, which induces the complete apoptosis of thymocytes (Fig. 3).

Fas system-mediated apoptosis of MC in the presence of actinomycin D or cycloheximide. According to the report indicating that the susceptibility of Fas system-mediated apoptosis increases in the presence of actinomycin D or cycloheximide (8), we cultured MC from MRL-+/+ or lpr/lpr mouse with anti-Fas Ab in the presence of actinomycin D or cycloheximide. As shown in Fig. 4, in the presence of 0.05μg/ml actinomycin D or 50 μg/ml cycloheximide, anti-Fas Ab induced apoptosis in MC from MRL-+/+ mice dose dependently upon anti-Fas Ab (Fig. 4A) but not in those from MRL-lpr/lpr mice (Fig. 4B, C). Further, in the presence of 0.5 μg/ml anti-Fas Ab, MC from MRL-+/+ mice dose dependently underwent apoptosis with actinomycin D (Fig. 4B) or cycloheximide (Fig. 4C). The MC from MRL-+/+ mouse cultured with anti-Fas Ab and actinomycin D for 20 hours showed DNA fragmentation, directly demonstrating the induction of apoptosis (Fig. 5).

mRNA expression of Fas ligand in MC. To investigate which cells regulate the Fas system of MC, we examined Fas ligand expression on MC by means of RT-PCR. Figure 6 shows that MC from MRL-+/+ or from MRL-lpr/lpr mouse cultured in 0.5% FBS expressed Fas ligand mRNA (Fig. 6). This result suggested that MC were, at least partly, autoregulated.

DISCUSSION

We studied whether the Fas system of MC contributes to the development or disease progression of MesGN in lpr mice and identified three key findings. MC cultured in vitro down-regulated Fas Ag expression, which was blocked by cycloheximide, showing a requirement of new protein

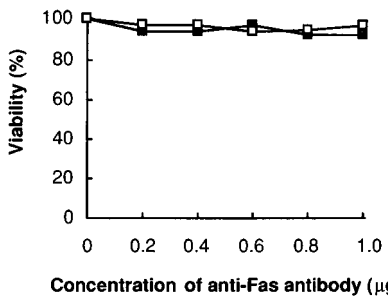

FIG. 3. A defective Fas-system mediated apoptosis with anti-Fas Ab alone. MC from MRL-+/+ (closed square) or MRL-lpr/lpr (open square) in media containing 0.5% FBS were cultured with anti-Fas Ab (0.5 μg/ml) for 20 hours. The viability was calculated as 100 × the viable cell counts between anti-Fas Ab-treated and untreated cells determined by trypan blue staining.

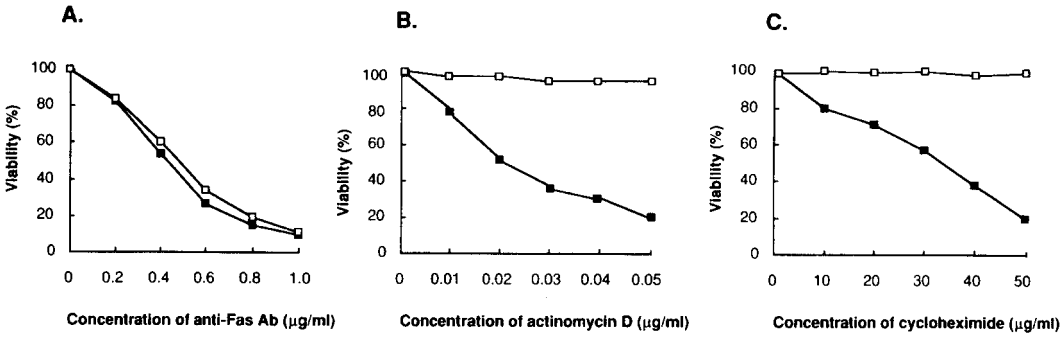

FIG. 4. Fas-system mediated apoptosis in the presence of actinomycin D or cycloheximide. (A) MC from MRL-+/+ mice in medium containing 0.5% FBS were cultured with various concentrations of anti-Fas Ab and 0.05 mg/ml actinomycin D (open square) or 50 mg/ml cycloheximide (closed square) for 20 hours. MC from MRL-+/+ (closed square) or MRL-lpr/lpr (open square) in media containing 0.5% FBS were cultured with anti-Fas Ab (0.5 μg/ml) and various concentrations of actinomycin D (B) or cycloheximide (C) for 20 hours. The viability was calculated as 100 × the viable cell counts between anti-Fas Ab-treated and untreated cells determined by trypan blue staining.

synthesis for this activity. In the absence of actinomycin D or cycloheximide, MC could not undergo Fas system-mediated apoptosis although Fas Ag was expressed at high levels. MC expressed mRNA for Fas ligand and Fas Ag. These findings suggest a possibility that defect in Fas system-mediated apoptosis cause proliferation of MC in vivo.

The expression intensity of Fas Ag on peripheral T cells was augmented when activated, increasing the susceptibility of these cells to Fas system-mediated apoptosis (9). In contrast, growing MC down-regulate this molecule and anti-Fas Ab alone could not direct these cells to undergo apoptosis despite their high level Fas Ag expression. These findings suggested that the regulatory mechanism and physiological role of the Fas system in MC differ from those of

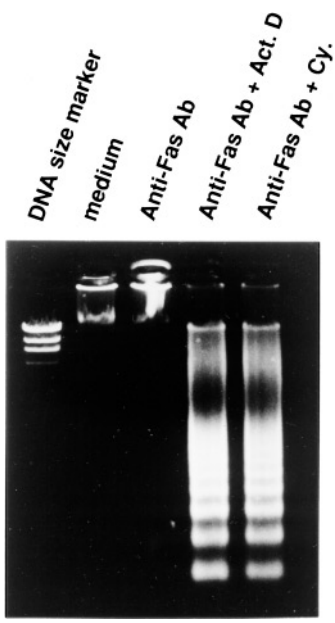

FIG. 5. Interchromosomal DNA fragmentation of MC upon cultured with anti-Fas Ab and actinomycin D. MC were cultured in the absence or presence of anti-Fas Ab (0.5 μg/ml) and actinomycin D (0.05 μg/ml) (Act.D) or cycloheximide (50 μg/ml) (Cy.) for 20 hours. Cell lysates were prepared as described in Materials and Methods. Fragmented DNA and intact chromatin were resolved on a 1.0% agarose gel and stained with ethidium bromide.

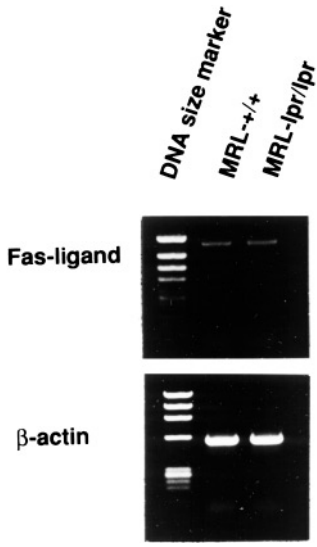

FIG. 6. Fas-ligand expression in MC. Total RNA of MC from MRL-+/+ or MRL-lpr/lpr mice cultured in media containing 0.5% FBS was extracted and RT-PCR was performed as described in Material and Methods. Hinc II-digested ϕX 174 DNA was used as the low molecular weight size marker.

peripheral T cells. MC underwent Fas system-mediated apoptosis in the presence of actinomycin D or cycloheximide, showing that a RNA or protein inhibiting this system exists in MC. The state of cells in which new synthesis of RNA or protein is inhibited seems to be applied for severely damaged or stressed states in vivo. Thus we infer that Fas system of MC removes useless cells and that during an early stage of inflammation, PDGF and other cytokines down-regulate the expression of Fas Ag to prevent apoptosis and to regulate inflammation. However, in the late stage of inflammation, severely damaged MC should be removed through the Fas system, since the synthesis of these inhibitory RNA or proteins is suppressed.

MC expressed mRNA of Fas ligand as well as Fas Ag. Although MC can interact with a variety of cells such as epithelial cells, infiltrated T cells and macrophages, the Fas system of MC should be at least partly autoregulated, as is the case of T cell regulation as we previously reported (10).

Baker et al. clearly showed that mesangial cell apoptosis contributes to the resolution of glomerular hypercellularity in an experimental nephritis model (11), although so far there is no study showing the contribution of apoptosis to human MesGN. As suggested in the present study, apoptotic pathway including Fas system of MC may contribute to the disease progression of human glomerulonephritis. We are investigating the role of Fas Ag and Fas ligand accompanied by other molecules related to apoptotic pathway in human MesGN.

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