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Suppression of Chemokine Gene Expression and Production in LPS-Stimulated Macrophages by a 130 kDa Glycoprotein from Plerocercoids of *Spirometra erinaceieuropae*i

Haruyo Matsuura, Asumi Tomioku, Satoko Kono, Sayuri Tademoto* and Soji Fukumoto

*Division of Medical Zoology, Department of Microbiology and Immunology, School of Medicine and *Technical Support Section, Tottori University Faculty of Medicine, Yonago 683-8503 Japan*

Previous studies have shown that excretory/secretory (ES) products from plerocercoids of *Spirometra erinaceieuropaei* have immunosuppressive activities. We report here that a 130 kDa glycoprotein (ES130) purified from ES products as a suppressive factor of nitric oxide synthesis in LPS-stimulated RAW 264.7 cells inhibited the gene expression of 3 chemokines, regulated on activation normal T cell expressed and secreted (CCL5/RANTES), macrophage inflammatory protein 2 (CXCL2/MIP-2), interferon-inducible protein 10 kDa (CXCL10/IP-10) in RAW 264.7 cells and mouse peritoneal macrophages stimulated with LPS for 3 h. These chemokines are important factors for recruitment of inflammatory leukocytes. RANTES acts on monocytes, basophils, lymphocytes, natural killer cells and eosinophils. MIP-2 is a potent chemoattactant for neutrophils, while IP-10 binds to Th1 cells. Nearly 80% of MIP-2 gene expression and 50% of IP-10 gene expression in peritoneal macrophages stimulated with LPS for 8 h was suppressed as well as these chemokine production by the preincubation with 100 ng/mL of ES130 or 5000 ng/mL crude ES products for 24 h. On the other hand the mRNA expression of RANTES in macrophages stimulated with LPS for 8 h or 24 h was not inhibited by ES130 or crude ES products, while the RANTES chemokine levels in the incubation medium were significantly suppressed. These results suggest that ES130 may attenuate inflammation around the plerocercoids by inhibiting these chemokine production.

Key words: macrophage; chemokine; excretory/secretory products; lipopolysaccharide; *Spirometra erinaceieuropaei*

The larval plerocercoids of *Spirometra erinaceieuropaei* are known to cause "sparganosis" in various tissues in the human (Kudesia et al., 1998). When the plerocercoids are taken orally in many mammals including humans or rodents, the head portion of the larva migrated into the peritoneal cavity from the intestines. These plerocercoids may have various bacteria on their surface and they bring these bacteria into the peritoneal cavity which, in turn, activates the peritoneal macrophages by the bacterial lipopolysaccharide (LPS). LPS is a ligand of Toll-like receptor 4, and has been known to be a potent activator of the immune system that induces local inflammation and

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ES, excretory/secretory; FBS, fetal bovine serum; IFN, interferon; IL-1, interleukin-1; ISRE, IFN-stimulated response element; IP-10, IFN-inducible protein 10 kDa; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor

septic shock (Rietschel and Brade, 1992; Guha and Mackman, 2001). It causes multiple effects on macrophages, including the secretion of chemokines for recruitment of inflammatory leukocytes and the inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α (Hsi and Remick, 1995).

The chemokines are divided into four families on the basis of the arrangement of the first 2 of 4 conserved cysteins (Ben-Baruch et al., 1995; Rottman, 1999). Many of the CXC chemokines are potent chemoattactants for neutrophils, but not monocytes, while CC chemokines act on monocytes, basophils, lymphocytes, natural killer cells and eosinophils (Wang et al., 1998; Rottman, 1999). In the mouse, the CXC family includes macrophage inflammatory protein 2 (CXCL2/MIP-2), interferon-inducible protein 10 kDa (CXCL10/IP-10) and KC (CXCL1/Gro-α). MIP-2 and KC bind to CXCR1 and /or CXCR2 expressed on neutrophils, while IP-10 binds to CXCR3 expressed on Th1 cells. The CC family includes JE/monocyte chemoattactant protein (CCL2/MCP-1) and regulated on activation normal T cell expressed and secreted (CCL5/RANTES). RANTES is a ligand for CCR1/3/5 expressed on monocytes, macrophages, T cells, dendritic cells and eosinophils (Wang et al., 1998; Rottman, 1999).

The experimentally infected plerocercoids do not induce the strong inflammatory responses around the parasites and can survive for long periods in the tissues of mice, hence we hypothesized that the larval plerocercoids of *S. erinaceieuropaei* secreted an immunosuppressive factor(s). We previously showed that the excretory/secretory (ES) products from the plerocercoids suppress the LPS-induced expressions of chemokines KC and JE (Fukumoto et al., 1997), TNF-α (Miura et al., 2001; Dirgahayu et al., 2002), and IL-1β (Dirgahayu et al., 2004) in murine macrophages.

In the present study, we purified an immunosuppressive factor (ES130) of a 130 kDa glycoprotein from ES products of plerocercoids and examined the effect on the chemokine gene expression.

Materials and Methods

Preparation of ES products from plerocercoids of Spirometra erinaceieuropaei

Plerocercoids of *Spirometra erinaceieuropaei* were collected from 2 species of snakes (*Elaphe quadrivirgata* and *Rhabdophis tigrinus*) in the southern part of Ehime Prefecture, Japan and stored for over 6 to 10 months in the subcutaneous tissue of golden hamsters, which were housed and maintained according to the guidelines for proper treatment of animals at the Research Center for Bioscience and Technology, Tottori University, Japan. ES products were obtained as described previously (Miura et al., 2001). To obtain ES products, 25 plerocercoids aseptically removed from hamsters were incubated for 24 h in 25 mL of Dulbecco's modified Eagle's medium (DMEM: Invitrogen, Carlsbad, CA) in a 10 cm dish. The medium was centrifuged at 10,000 × *g* for 30 min at 4°C to remove insoluble debris, then dialyzed against 25 mM Tris-HCl (pH 7.4) and concentrated with Amicon Ultra-15 Centrifugal Filter Units (Nihon Millipore, Tokyo, Japan), and subsequently sterilized with a 0.22 μm filter (Nihon Millipore). The ES products after sterilization are referred to as crude ES products hereafter. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and adjusted to 500 μg/mL for culture or 250 μg/mL for purification.

Purification of an immunosuppressive factor from ES products

The crude ES products were loaded onto a new MonoQ HR5/5 anion-exchange column (Amersham Bio Sciences, Piscataway, NJ) equilibrated in 25 mM Tris-HCl (pH 7.4), and the column was washed with 10 column volumes of same buffer. The bound materials were eluted with a step gradient consisting of 10 column volumes each of 25 mM Tris-HCl (pH 7.4) containing 0.1, 0.2, 0.3, 0.4,

0.5 M NaCl. These samples were determined for their inhibitory effect on the nitrite production in RAW 264.7 macrophages (RCB00535) that were stimulated with LPS (*Escherichia coli* serotype 005:B5) (Difco Laboratories, Detroit, MI). The strongest inhibition was obtained in the fraction eluted with Tris-HCl containing 0.3 M NaCl. For further purification, the fractions eluted with 0.3 M NaCl were applied on *Ricinus communis* agglutinin (RCA120)- and wheat germ agglutinin (WGA)-agarose column (Seikagaku, Tokyo) sequentially according to the manufacture's instructions. To measure the protein concentration, the purified proteins and bovine serum albumin (Sigma Aldrich, St. Louis, MI), as a standard protein, were applied to 5% to 20% linear gradient polyacrylamide gel (SPG-R520L; ATTO, Osaka, Japan) for SDS-PAGE. After that, the polyacrylamide gel was stained with SYPRO Ruby gel staining (Invitrogen), and the concentration of the purified proteins was quantified by densitometry (ATTO Densitograph 4.0; ATTO).

Preparation and culture of macrophage

RAW 264.7 cells were cultured according to Dirgahayu et al. (2004). Male C57/BL6 mice (8-week-old) were purchased from Japan SLC (Shizuoka, Japan). These mice were housed and maintained at the Research Center for Bioscience and Technology, Tottori University. Peritoneal macrophages were harvested with 10 mL of ice-cold phosphate-buffered saline on the 3rd day after intraperitoneal injection of 2 mL thioglycolate (Sigma-Ardrich). The macrophages in DMEM containing penicillin G (Banyu Pharmaceutical, Tokyo), streptomycin (Meiji Seika, Tokyo) and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen,) were plated in 60 mm tissue culture dish (Greiner, Bayern, Germany). Then the cells were incubated at 37˚C in an atmosphere of 5% CO_2 and were left for more than 14 h before each experiment.

The culture medium was replaced with a fresh DMEM including penicillin G, streptomycin and 10% FBS, and either with crude ES products (0.5–5.0 μg/mL) or purified ES products (10–200 ng/mL). The cells were incubated for 24 h, and were stimulated with 100 ng/mL LPS. After 3 h, 8 h or 24 h of incubation, the cells were collected for isolation of RNA, and the supernatant fluids of these dishes were collected for the measurement of chemokines by enzyme-linked immunosorbent assay (ELISA).

RNA isolation and semi-quantitative reverse transcription-PCR

Total RNA was prepared using an ISOGEN Kit (Nippon Gene, Tokyo) according to the manufacture's protocol. Total RNA (1 μg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Tokyo) and random primers. The cDNA was subjected to PCR amplification with *Taq* DNA polymerase (Gene *Taq*) (Nippon Gene). The primers for IL-1β and TNF-

Table 1. Chemokine primers and cycle number of PCR

Gene	Primer		Product size (bp)	Cycle
CCL5/RANTES	*5′-CCT CAC CAT CAT CCT CAC TGC A-3′*	(forward)	334	34
	5′-CAT CCC CAA GCT GGC TAG GAC T-3′	(reverse)		
CXCL2/MIP-2	*5′-AGT TTG CCT TGA CCC TGA AGC C-3′*	(forward)	466	28
	5′-TGG GTG GGA TGT AGC TAG TTC C-3′	(reverse)		
CXCL10/IP-10	*5′-CCT ATC CTG CCC ACG TGT TGA G-3′*	(forward)	431	23
	5′-CGC ACC TCC ACA TAG CTT ACA G-3′	(reverse)		
β-actin	*5′-TGG AAT CCT GTG GCA TCC ATG AAA-3′*	(forward)	349	22
	5′-TAA ACC GCA GCT CAG TAA CAG TCC-3′	(reverse)		

IP-10, interferon-inducible protein 10 kDa; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted.

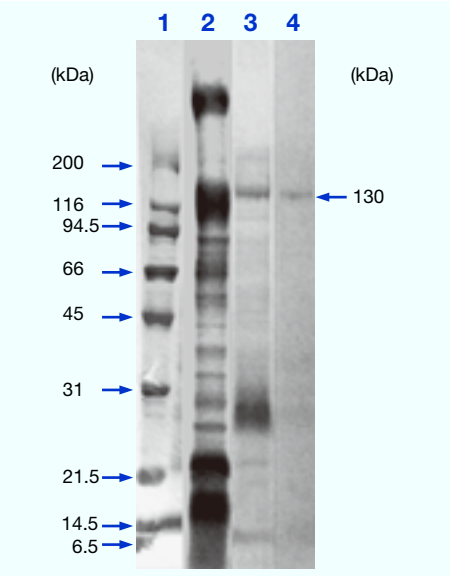

Fig. 1. An immunosuppressive factor, ES130, purified with ion-exchange chromatography and lectin chromatography was analyzed by SDS-PAGE with SYPRO Ruby gel staining. Lane 1, molecular marker; lane 2, crude ES products; lane 3, the fraction eluted from the MonoQ column with Tris-HCl containing 0.3 M NaCl; lane 4, the fraction eluted from the RCA120- and WGA-agarose columns sequentially. ES, excretory/secretory; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin.

α were used according to Kina et al. (2005), and those for cyclooxygenase-2 (COX-2) were based on Fukumoto et al. (2006). The primers that we used for RANTES (Heeger et al., 1992), MIP-2 (Su et al., 1996), IP-10 (Baker et al., 2003) and β -actin (Alonso et al., 1986) are listed in Table 1. The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 1min. The number of cycles for semi-quantitative reverse transcriptase-PCR (RT-PCR) of each gene was also listed in Table 1. The PCR products were separated by electrophoresis on 1.5 % agarose gels, and stained with ethidium bromide and photographed under UV light with a digital camera. For quantification, electrophoretic gels were analyzed using the software Image J version 1.33 (National Institutes of Health Bethesda, MD). The amount of mRNAs was normalized to that of β-actin.

ELISA analysis

The culture medium of 4.0×10^6 macrophages in 60 mm dishes was harvested after incubation with LPS for 8 h or 24 h. The collected supernatants were stored at −80˚C before measurement. The concentrations of RANTES, MIP-2 and IP-10 were assayed using ELISA kit (R & D Systems, Minneapolis, MN), following the manufacturer's protocol.

Statistical analysis was performed by non-repeated measures analysis of variance. If there was significant difference between the positive control and treatment groups, Dunnett's test was subsequently performed. Data were analyzed with statistical package software SPSS 12.0 for Windows.

Results

Purification of an immunosuppressive factor (ES130)

A suppressive effect on NO synthesis in LPS-stimulated RAW 264.7 cells was found in 0.3 M NaCl fraction of crude ES products by an anion exchange chromatograpy. Then we purified a glycoprotein of 130 kDa, which suppressed NO synthesis in RAW 264.7 cells, using 2 lectin columns, RCA-agarose- and WGA-agarose-columns sequencially (Fig. 1).

Suppression of chemokine mRNA expression in RAW 264.7 cells by ES130

Preincubation with 100 ng/mL of ES130 for 24 h suppressed the mRNA expression of 3 proinflammatory genes, IL-1β, TNF-α and COX-2, and 3 chemokine genes, MIP-2, RANTES and IP-10 as well as 5 μg/mL of crude ES in RAW 264.7 cells stimulated with LPS for 3 h (Fig. 2).

Suppression of chemokine mRNA expression in peritoneal macrophages by ES130

No appreciable expression of chemokines was observed in control macrophages without LPS

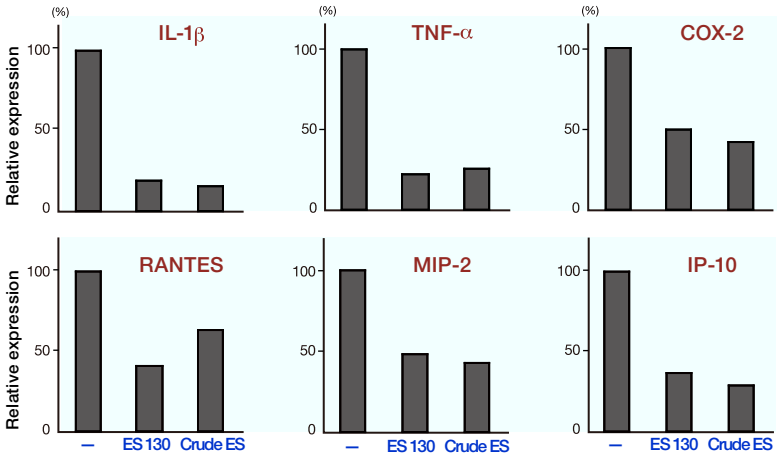

Fig. 2. ES130 inhibited the mRNA expression of proinflammatory genes including 3 chemokines in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were left untreated or incubated with ES130 (100 ng/mL) or crude ES products (5 μg/mL) for 24 h, then stimulated with LPS (100 ng/mL) for 3 h. Total RNA was obtained and the mRNA expression of IL-1β, TNF-α, COX-2, MIP-2, RANTES, IP-10 and β-actin was analyzed by semi-quantitative RT-PCR. The mRNA levels of proinflammtory genes were quantified and were normalized to β-actin. Relative mRNA levels of these genes were presented as percentages of the LPS-induced control in the absence of ES130 or crude ES products. ES, excretory/secretory; IL-1, interleukin-1; IP-10, interferon-inducible protein 10 kDa; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase.

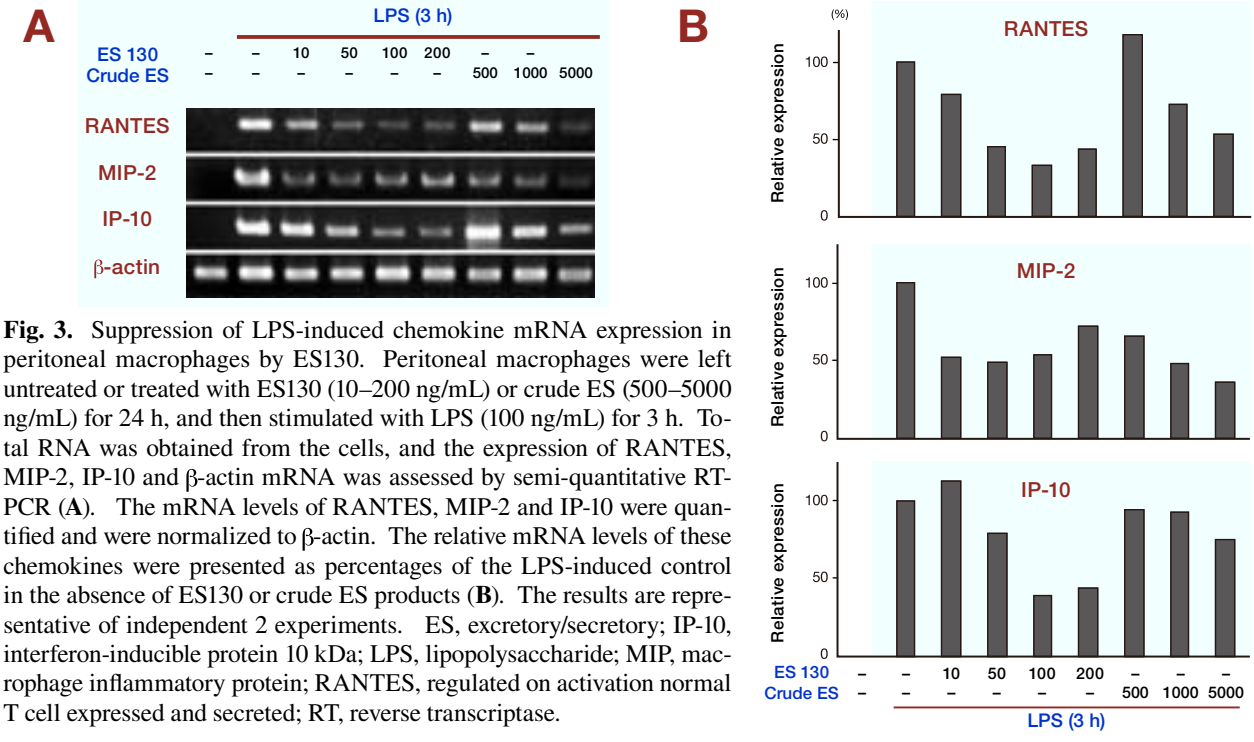

Fig. 3. Suppression of LPS-induced chemokine mRNA expression in peritoneal macrophages by ES130. Peritoneal macrophages were left untreated or treated with ES130 (10–200 ng/mL) or crude ES (500–5000 ng/mL) for 24 h, and then stimulated with LPS (100 ng/mL) for 3 h. Total RNA was obtained from the cells, and the expression of RANTES, MIP-2, IP-10 and β-actin mRNA was assessed by semi-quantitative RT-PCR (**A**). The mRNA levels of RANTES, MIP-2 and IP-10 were quantified and were normalized to β-actin. The relative mRNA levels of these chemokines were presented as percentages of the LPS-induced control in the absence of ES130 or crude ES products (**B**). The results are representative of independent 2 experiments. ES, excretory/secretory; IP-10, interferon-inducible protein 10 kDa; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase.

stimulation. Mouse peritoneal macrophages were either left untreated or preincubated with various doses of ES130 or crude ES products for 24 h. The cells were stimulated with 100 ng/mL LPS for 3 h. Crude ES products suppressed 3 chemokine gene expression in a dose-dependent manner. Ten to 100 ng/mL of ES130 also suppressed RANTES and IP-10 in dose-dependent manner, while about

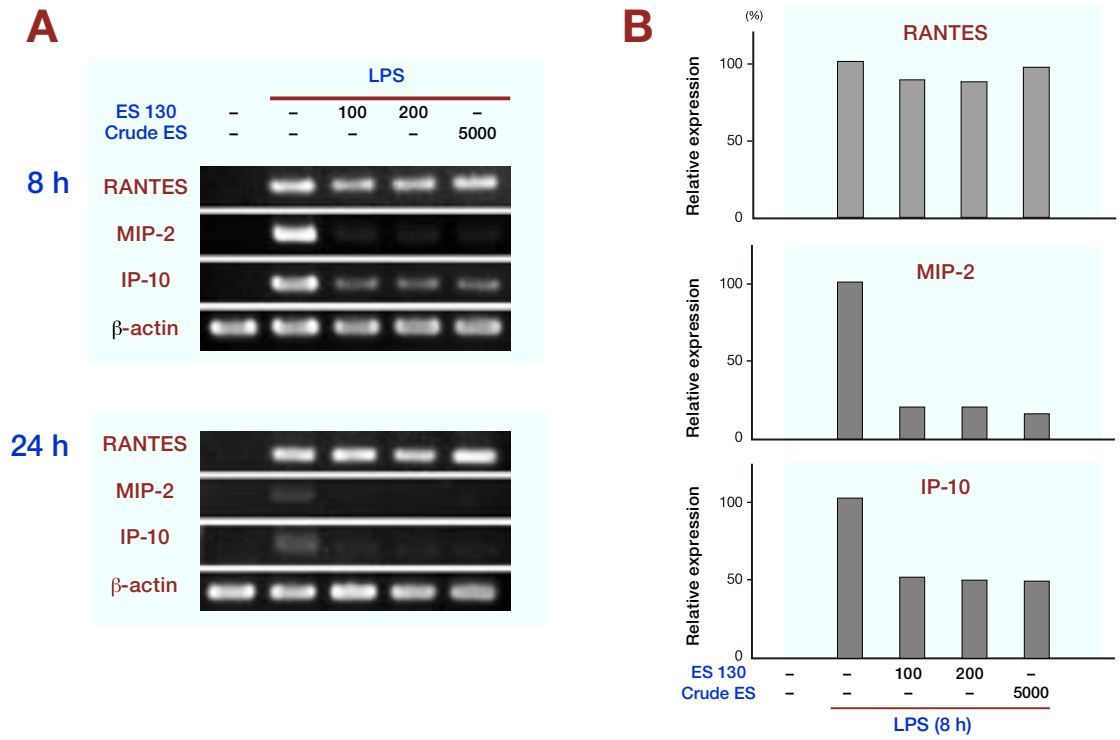

Fig. 4. Suppression of LPS-induced chemokine mRNA expression in murine peritoneal macrophages by ES130. Peritoneal macrophages were left untreated or treated with ES130 (100 or 200 ng/mL) or crude ES (5000 ng/mL) for 24 h, and then stimulated with LPS (100 ng/mL) for 8 h or 24 h. Total RNA was obtained from the cells, and the mRNA expression of RANTES, MIP-2, IP-10 and β-actin was assessed by semi-quantitative RT-PCR (**A**). The mRNA levels of RANTES, MIP-2 and IP-10 in macrophages stimulated with LPS for 8 h were quantified and were normalized to β-actin. The relative mRNA levels of these chemokines were presented as percentages of the LPS-induced control in the absence of ES130 or crude ES products (**B**). The results are representative of 2 independent experiments. ES, excretory/secretory; IP-10, interferon-inducible protein 10 kDa; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase.

half of the MIP-2 gene expression was inhibited by 10 to 100 ng/mL of ES130 (Fig. 3). However, 200 ng/mL of ES130 did not inhibit the gene expression of 3 chemokines more than the dose of 100 ng/mL.

Nearly 80% of MIP-2 gene expression and 50% of IP-10 gene expression in peritoneal macrophages stimulated with LPS for 8 h were suppressed by the preincubation with 100 or 200 ng/mL of ES130 or 5000 ng/mL crude ES products for 24 h. The suppressive effect on the gene expression of MIP-2 and IP-10 continued in macrophages after stimulation with LPS for 24 h. On the other hand, the mRNA expression of RANTES in macrophages stimulated with LPS for 8 h or 24 h was not inhibited by ES130 or crude ES products (Fig. 4).

Suppressive effect of ES130 on chemokine production in the medium of macrophages

The effects of ES130 and crude ES products on 3 chemokine leves in the incubation medium of peritoneal macrophages stimulated with LPS for 8 h and 24 h were examined by ELISA.

MIP-2 concentrations in the medium of LPS-stimulated macrophages for 8 h and 24 h were deeply suppressed by ES130 or crude ES products (Fig. 5) as well as the suppression of MIP-2 mRNA levels (Fig. 4). The IP-10 concentration in the medium of macrophages stimulated for 8 h or 24 h was also suppressed by ES130 or crude ES products significantly (Fig. 5).