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Interleukin-6 production by peritoneal mesothelial cells and its regulation by inflammatory factors in rats administered carbon tetrachloride intraperitoneally

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Abstract

We previously reported that a high level of interleukin-6 (IL-6), which is protective against CCl_4-induced hepatotoxicity, is produced in the peritoneal cavity in the early period after ip carbon tetrachloride (CCl_4) administration. The objective of this study was to identify the tissues and cells involved in IL-6 production and clarify the mechanisms underlying its regulation. IL-6 mRNA levels increased significantly in the serous membranes of the mesentery and peritoneum, but not in the parenchymal organs including liver, kidney and spleen, 3 h after ip CCl_4 administration. Peritoneal mesothelial cells (PMCs), a major cell population in serous membranes, were isolated from rat peritoneal walls by trypsin digestion and cultured with peritoneal exudate fluid (PEF) from CCl_4-administered rats. PMCs produced a high level of IL-6 in the presence of PEF recovered 0.5 h after ip CCl_4 administration. Analyses of PEF revealed that the levels of prostaglandin E_2 (PGE_2), histamine, IL-1α, IL-1β and tumor necrosis factor-α (TNF-α) increased immediately after ip CCl_4 administration. These inflammatory factors, except for histamine, stimulated IL-6 production to varying degrees, in the following order: IL-1α > IL-1β > TNF-α ≫ PGE_2.

In summary, the present study indicates that the high level of IL-6 observed in the rat peritoneal cavity after ip CCl_4 administration is at least partially produced by PMCs stimulated cooperatively with IL-1α, IL-1β, TNF-α and PGE_2. These inflammatory factors may be released from tissues or cells either stimulated or injured directly by CCl_4.

Keywords: Interleukin-6; Carbon tetrachloride; Peritoneal cavity; Mesothelial cell; Inflammatory factor

Introduction

Carbon tetrachloride (CCl_4) is a model compound for the study of hepatotoxicity. It is activated in the liver by cytochrome P450 (2E1, 2B1/2B2) to form a highly reactive trichloromethyl radical and then a trichloromethyl peroxy radical. These radicals bind to cellular molecules such as nucleic acids, proteins and lipids, and adversely affect the permeability of mitochondria, endoplasmic reticulum and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis, which largely contributes to the subsequent hepatotoxic damage (Plaa, 2000; Weber et al., 2003). In animal models, CCl_4 is administered by different routes and at various vehicle-to-CCl_4 ratios (Janakat and Al-Merie, 2002). Since CCl_4 shows solvent toxicity (Berger et al., 1986) when it is administered via invasive routes, such as ip and sc, at a low vehicle-to-CCl_4 ratio, a number of inflammatory responses, including cytokine production, may be caused at the site of contact (Heinrich et al., 1990).

Interleukin-6 (IL-6) is a pleiotropic inflammatory cytokine (Barton, 1996) that plays an important role in several processes involved in hepatic physiology (Taub, 2004), including liver regeneration (Cressman et al., 1996), acute-phase responses (Heinrich et al., 1990) and hepatoprotection (Kovalovich et al., 2001; Haga et al., 2003). The protective roles of IL-6 against CCl_4-induced hepatotoxicity have been shown by the findings that IL-6 knockout mice are subject to more intense liver injury and lipid peroxidation than wild-type mice upon CCl_4 administration (Katz et al., 1998; Kovalovich et al., 2000). Moreover, IL-6 administration before CCl_4 administration inhibits

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CCl_4-mediated apoptosis and accelerates hepatocyte regeneration in the livers of both IL-6 knockout and wild-type mice (Kovalovich et al., 2000). We observed that IL-6 pretreatment of rats significantly suppresses liver enzyme release and lipid peroxidation induced by CCl_4 administration (Sato et al., 1995).

We previously observed that IL-6 levels are increased in the early period after CCl_4 administration via sc and ip routes, but not via a po route, in rats, and that the amount of IL-6 in the peritoneal cavity into which CCl_4 was administered increased 37-fold compared with the plasma level. These results suggested that IL-6 is locally produced in tissues or cells in the peritoneal cavity, contrary to the systemic IL-6 production caused by a well-known IL-6 inducer, lipopolysaccharide (LPS) (Hojo et al., 2002; Zuinen et al., 2007). In the present study we searched for the tissues and cells involved in IL-6 production in the peritoneal cavity and analyzed the mechanisms of IL-6 regulation by inflammatory factors in rats administered CCl_4 intraperitoneally.

Materials and methods

Reagents. Highly pure grade CCl_4 (99.9%), corn oil, recombinant rat (rr) IL-1α, rr IL-1β, rr tumor necrosis factor-α (TNF-α) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Prostaglandin E_2 (PGE_2) was purchased from Sigma-Aldrich (St. Louis, MO) and rr IL-6 was purchased from Pepro Tec EC (London, UK).

Animals and treatment. Male Wistar rats (4–6 weeks) were purchased from SLC Japan (Shizuoka, Japan). The animals were housed for at least 1 week in an environmentally controlled room (temperature, 23±2 °C; humidity, 55±5%; 12-h dark/light cycle) with access to food and water *ad libitum* before use. They were administered CCl_4 intraperitoneally at 1.0 ml/kg body weight in 50% (v/v) sterile corn oil or an equivalent volume of corn oil as a control, unless otherwise mentioned. All animal studies were carried out with permission from the Ethical Committee for Animal Welfare of Showa Pharmaceutical University, Tokyo, Japan.

IL-6 mRNA expression in various tissues after ip CCl_4 administration. IL-6 mRNA expression in tissues was determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The control group received an equal volume of corn oil. The liver, kidney, spleen, mesentery and peritoneum were removed from rats 3 h after CCl_4 or corn oil administration. The capsules of the spleen and kidney were removed and the peritoneum was detached carefully from other tissues using forceps and scissors. The tissues were stored at −80 °C until use. Total RNA was isolated from frozen tissues using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA was prepared from total RNA samples using a SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). An aliquot (0.5 μl) of each RT reaction mixture was used for PCR using reagents purchased from Takara Bio Inc. (Shiga, Japan). The PCR products were separated by 10% polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide and scanned. The intensities of the bands were measured using Image J 1.34 (National Institutes of Health). The results were expressed as the ratio of IL-6/β-actin. The PCR primers used in this study were as follows: IL-6 (5′-TAGAGTCACAGAAGGAGTGG-3′ and 5′-GCCAGTTCTTCGTAGAGAAC-3′) and β-actin (5′-CAATGTGGCTGAGGACTTTG-3′ and 5′-ACAGAAGCAATGCTGTCACC-3′).

Isolation of peritoneal mesothelial cells (PMCs). PMCs were isolated from the peritoneal walls of 5-week-old male Wistar rats using the trypsin/ethylenediaminetetraacetic acid (EDTA) digestion method described by Tamura et al. (2003) with a slight modification. Briefly, an aseptically detached peritoneal tissue was placed on the cap of a 15- or 50-ml centrifuge tube that was placed inversely in a 100-mm dish and pressed gently at the center of the tissue to form a hollow. Then, a small amount of warm 0.25% trypsin/1 mM EDTA solution (GIBCO, Carlsbad, CA) was poured into the hollow and the tissue was incubated at 37 °C for 30 min. The recovered fluid was centrifuged at 1500 rpm for 5 min; the resulting cell pellet was suspended in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS, JRH Biosciences A CSL Co., Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured in 5% CO_2 at 37 °C. Experiments were performed using PMCs at the 3rd passage.

Immunocytochemistry of PMCs. PMCs were identified by their cobblestone appearance and a positive reaction with a monoclonal antibody to cytokeratin (Connell and Rheinwald, 1983). Briefly, PMCs were placed in an eight-well chamber slide (Nalge Nunc, Naperville, IL) at a concentration of 2×10^5 cells/ml, at 0.5 ml per chamber, and cultured up to 80% confluence. The cultured cells were washed in cold PBS, fixed in cold acetone–methanol (1:1, v/v) for 5 min and the endogenous peroxidase activity was quenched by incubating the cells in 0.3% (v/v) H_2O_2 for 10 min. The cells were then incubated with a mouse anti-keratin/cytokeratin monoclonal antibody (422431, IgG1, Nichirei Biosciences Inc., Tokyo, Japan) or monoclonal antibodies (mouse IgG1) to non-mammalian antigens (X0931, DAKO, Carpinteria, CA) as negative controls, for 1 h. The slides were further incubated with horseradish peroxidase-conjugated anti-mouse IgG (DAKO) for 45 min. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride and cells were counterstained with hematoxylin. The cells were dehydrated through a graded alcohol series and finally xylene, and cover-slipped using a permanent mounting medium. Cells stained with the specific antibody were observed under a microscope.

Preparations of PEF and peritoneal exudate cells (PECs). Experimental and control rats were euthanized under ether anesthesia and then intraperitoneally injected with 10 ml of RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.) containing 1% FBS and 100 U of heparin, after which they were massaged lightly on the abdomen. The peritoneal fluid was aspirated and centrifuged at 1500 rpm for 5 min and the supernatant was centrifuged at 12,000 rpm for 10 min. The resultant supernatant (PEF) was stored at −80 °C until use. For stimulation of PMCs, a mixture of PEF from five rats was sterilized through a 0.22 μm Millipore filter. Precipitated cells and those obtained from the washing of the peritoneal cavity were combined, washed and suspended in medium for counting of cell number.

Preparation of the culture supernatants of PMCs stimulated with PEF and inflammatory factors. PMCs (1×10^5 cells/well in 24-well plates) were precultured for 48 h and then cultured with PEF diluted in serum-free DMEM or stimulants (specifically, PGE_2, histamine, IL-1α, IL-1β and TNF-α) for 24 h.

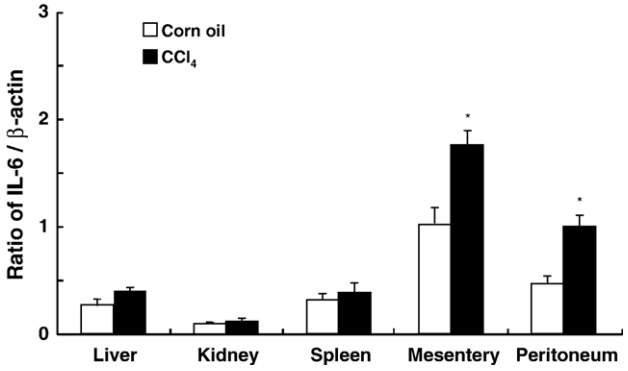

Fig. 1. Effect of ip CCl_4 administration on IL-6 mRNA expression level in tissues in the peritoneal cavity. Rats were administered CCl_4 intraperitoneally at 1.0 ml/kg body weight in 50% (v/v) corn oil. The control group received an equivalent volume of corn oil. The liver, kidney, spleen, mesentery and peritoneum were collected 3 h after administration. The expression levels of IL-6 and β-actin mRNA were determined by RT-PCR analysis and the band densities were analyzed using Image J 1.34. Data are expressed as the means±S.E. of at least three independent experiments. Significantly different from control; $*p<0.05$.