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Involvement of Glial Cell Line-Derived Neurotrophic Factor in Activation Processes of Rodent Macrophages

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The physiological roles of glial cell line-derived neurotrophic factor (GDNF) expressed in the microglia/macrophages of the injured spinal cord have not yet been clarified. mRNA expression of chemokines, including monocyte chemoattractant protein (MCP)-1, was evoked within 1 hr after transection of the spinal cord, and GDNF mRNA expression was similarly up-regulated. Immunohistochemical analysis showed that GDNF was coexpressed with MCP-1 in the CD11b-positive cells. Therefore, we examined further the effects of GDNF on cultured rat peritoneal macrophages. GDNF enhanced the phagocytic activity of the macrophages via GFRα-1, glycosylphosphatidylinositol-anchored specific binding site of GDNF, in a c-Ret-independent manner. The influence of autocrine and/or paracrine GDNF synthesis was evaluated by performing activation experiments using macrophages cultured from heterozygous (+/–) GDNF gene-deficient mice or wild-type (+/+) mice. There were no morphological differences dependent on genetic types or stimulators. However, the GDNF mRNA level, but not the MCP-1 or GFRα-1 mRNA level, was substantially lower in the mutant macrophages than in the +/+ cells irrespective of stimulation with MCP-1 or lipopolysaccharide (LPS). The phagocytic activity enhanced by MCP-1 or LPS was significantly lower in the mutant cells (+/–) than in the +/+ ones, demonstrating the involvement of endogenous GDNF in the activation processes of macrophages in vitro and suggesting that not only neuroprotective function but also activation of macrophages is effected by the GDNF produced after a spinal cord injury. © 2005 Wiley-Liss, Inc.

Key words: macrophages; phagocytic activity; spinal cord; glial cell line-derived neurotrophic factor; GDNF; monocyte chemoattractant protein-1; MCP-1

Several experimental strategies have been employed to minimize tissue damage and to enhance axonal growth and regeneration after injury to the central nervous system (CNS). The failure of axonal regeneration after CNS injury is thought to result in part from the expression of molecules inhibitory for axonal growth (Fawcett and Asher, 1999), the lack of neurotrophic factors (Widenfalk et al., 2001), and/or inflammatory reactions (Franzen et al., 1998).

Inflammation is one of the responses occurring immediately after spinal cord injury and is likely to cause secondary injury that magnifies the primary injury and facilitates neuronal dysfunction. This inflammatory response is characterized by the invasion of leukocytes into the injury site and the activation of resident glial cells, which is regulated by chemokines in the case of various injuries and diseases of the CNS (Glabinski et al., 1995; Adams and Lloyd, 1997; Rollins, 1997). Several studies have shown that accumulation of macrophages around the lesion site is correlated with the secondary injury that appears after a spinal cord injury (Blight, 1985, 1992; Popovich et al., 1997; Zhang et al., 1997). On the other hand, inflammation is also thought to contribute to the induction of neurotrophic factors beneficial to damaged neurons. It has been shown that glial cell line-derived neurotrophic factor (GDNF), one of the most potent neurotrophic factors for motoneurons (Lin et al., 1993; Henderson et al., 1994), is expressed in leukocytes and especially in activated microglia/macrophages of the injured brain (Batchelor et al., 1999; Wei et al., 2000). There is also a report showing that the activated microglia/macrophages in the injured spinal cord express GDNF (Satake et al., 2000; Widenfalk et al., 2001). How-

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ever, the interaction between neurotrophic factors and chemokines in the injured spinal cord and the physiological significance of GDNF expression in microglia/macrophages have not been studied.

In this study, we found that 1) microglia/macrophages recruited into the transected spinal cord coexpressed both GDNF and one of the chemokines, monocyte chemoattractant protein (MCP)-1; 2) cultured peritoneal macrophages responded to exogenous GDNF by enhancing their phagocytic activity and expression of MCP-1 mRNA; and 3) macrophages cultured from the heterozygous (+/–) GDNF gene-deficient mice were activated by MCP-1 or lipopolysaccharide (LPS) to a much lesser extent than those cultured from the +/+ mice. These results suggest that GDNF induced in activated macrophages behaves as a cytokine for macrophages and participates in the development of inflammation in the injured CNS in addition to functioning in its role as a neurotrophic factor.

MATERIALS AND METHODS

Animals and Surgery

Animals were cared for according to the guidelines for experimental animal care issued from the Office of the Prime Minister of Japan. Adult male Wistar rats (7–8 weeks old, 150–200 g; Nippon SLC, Shizuoka, Japan) were anesthetized by an intraperitoneal injection with pentobarbital (30 mg/kg). After laminectomy at the level of the ninth thoracic vertebra, the exposed spinal cords were completely transected with a razor blade. Afterward, the incision sites were sutured. The generation of the GDNF gene-deficient mice has been described elsewhere (Picher et al., 1996).

Reverse Transcription-Polymerase Chain Reaction

The spinal cords were dissected out from the anesthetized animals 0, 1, 3, 6, 12, 24, or 48 hr after the spinal cord transection, and segments (5 mm in length) of each spinal cord just rostral or caudal to the transected site were collected and pooled. RNA was prepared from the collected tissues by using Isogen (Nippon Gene, Tokyo, Japan), which is basically composed of guanidine isothiocyanate. The reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to the instructions. In brief, 500 ng of RNA was denatured for 2 min at 70°C and reverse transcribed with MMLV reverse transcriptase for 1 hr at 42°C in the presence of 1 μM CDS primer. Afterward, the transcriptase was inactivated by heating for 7 min at 72°C. The synthesized cDNA was amplified with each pair of primers by PCR. The amplification was carried out with the thermal cycler at 94°C for 3 min, followed by 20–36 cycles consisting of 94°C for 30 sec, 52–60°C for 30 sec, and 72°C for 30 sec. The products were electrophoresed on 2% agarose gels. The predicted sizes of amplified cDNA fragments, the numbers of PCR cycles, and the annealing temperatures are indicated in Table I. β-Actin mRNA was used as an internal control. The intensity of the bands was analyzed by use of image-analysis software (Mac BAS; Fuji Film, Tokyo, Japan). RNA from rat peritoneal macrophages cultured in the presence of GDNF, MCP-1 (PreproTech, Rocky Hill, NJ), or LPS (Sigma, St. Louis, MO) was prepared and analyzed by RT-PCR as described above.

Immunohistochemical Study

Two days after the spinal cord transection, animals were anesthetized with pentobarbital (30 mg/kg) and then cardioperfused with cold 4% (w/v) paraformaldehyde solution prepared in 0.1 M phosphate buffer, pH 7.3 (the fixative). The spinal cord was postfixed with the same fixative for 2 hr, soaked in 20% (w/v) sucrose for 1 day, and frozen in embedding compound (Tissue-Tec; Miles, Elkhart, IN). Coronal sections of 30 μm thickness were cut with a cryostat (model CM 1800; Leica, Deerfield, IL) and thawed on coverslips bearing covalently linked amino groups (Sumitomo Bakelite, Tokyo, Japan). The tissue sections were next dried and subsequently soaked in the fixative for 5 min to cross-link covalently the tissue sections to the coverslips. Then, they were rinsed in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.3% (v/v) Triton X-100 (TT buffer) at 37°C for 30 min to make the cell membrane permeable to antibodies. Next, the sections were incubated with TT buffer containing anti-GDNF rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution or anti-MCP-1 rabbit antibody (Biogenesis, Poole, United Kingdom) at a 1:1,000 dilution or antiglial fibrillary acidic protein mouse antibody (GFAP; Boehringer Mannheim Biochemica, Indianapolis, IN), anti-CD11b mouse antibody (Chemicon, Temecula, CA), and anti-NeuN mouse antibody (Chemicon) at a 1:1,000 dilution at 4°C overnight, and subsequently incubated at room temperature for 2 hr with TT buffer containing both fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG and rhodamine-labeled anti-mouse IgG antibodies (Chemicon). After having been washed with TT buffer, the sections were observed for fluorescence with a confocal laser microscope (Zeiss model L SM510).

Cell Cultures

Macrophages were obtained from the peritoneal cavity of adult male Wistar rats or adult GDNF gene-deficient mice [wild-type mice (+/+) or heterozygous mice (+/–)] 2 days after an intraperitoneal injection of 200 μg of concanavalin A (Sigma) in 5 ml phosphate-buffered saline (PBS). The cells were washed with PBS and cultured in RPMI 1640 medium (Sigma) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Cansera Int., Rexdale, Canada); 1 hr later, the medium was aspirated to remove nonadherent cells and replaced with fresh medium.

Phagocytosis Assay

The FITC-labeled latex beads (1.0 μm in diameter; Polysciences, Warrington, PA) were opsonized by incubation with human IgG (Sigma) at 4°C overnight, washed, and suspended in PBS. Cells pretreated or not with phosphatidylinositol-specific phospholipase C (PI-PLC, 0.5 U/ml; Sigma) for 1 hr were cultured in the presence of GDNF, MCP-1, or LPS and then incubated in the medium containing 0.01% FITC-labeled latex beads for 30 min at 37°C. Excess beads were washed away with PBS, and incorporated beads were visualized with a fluorescence

TABLE I. Sequences of Oligonucleotide Primers Used in the RT-PCR

Gene	Upstream primer	Downstream primer	Product size (bases)	Annealing temperature (°C)	Cycle number
GDNF	GAGAGGAATCGGCAG GCTGCAGCTG	CAGATACATCCACAT CGTTTAGCGG	337	60	36
NGF	GGGGATCCTCCACCC ACCCAGTCTTCCAC	GGCAAGTCAGCCTCT TCTTGTAGCCTTCC	376	60	30
BDNF	GGAATTCGAGTGATG ACCATCCTTTTCCTTAC	CGGATCCCTATCTTC CCCTTTTAATGGTCAGTG	771	60	31
NT-3	TATGCAGAACATAAG AGTCAC	CTACGAGTTTGTTGT TTTCTG	294	55	36
MCP-1	TAGCATCCACGTGCT GTCTC	CATTCAAAGGTGCTG AAGTCC	299	55	25
MIP-1α	TGCCCTTGCTGTTCT TCTCT	AGATCTGCCGGTTTC TCTTG	200	55	30
MIP-1β	CTCTCTCCTCCTGCT TGTGG	CACAGATTTGCCTGC CTTTT	200	55	30
MIP-2	AGGGTACAGGGGTTG TTGTG	TTTGGACGATCCTCT GAACC	204	55	30
RANTES	ATATGGCTCGGACAC CACTC	CCCACTTCTTCTCTG GGTTG	167	55	30
CCR2	CTGCCCCTACTTGTC ATGGT	AACGCAGCAGTGTGT CATTC	264	55	30
GFRα-1	TCCAGCCACATAACC ACAAA	CTTCAACAGAAGCCC CTGAG	297	55	30
c-Ret	CGGCACACCTCTGCT CTATG	CTGGAGGAAGACGGT GAGCA	237	55	35
β-Actin	GTGGGCCGCCCTAGG CACCAG	CTCTTTAATGTCACG CACGAT	542	55	32

microscope. Phagocytic activity was quantified as the number of FITC-positive cells (phagocytosing macrophages) per total cells.

RESULTS

Injury-Induced mRNA Expression of Neurotrophic Factors

Time-dependent mRNA expression of GDNF or neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), was examined in the segment adjacent to the transection site of the rat spinal cord (Fig. 1). NGF, BDNF, and GDNF mRNA levels were significantly elevated after the injury, but the NT-3 mRNA level remained constant throughout the experimental period. The increase was greatest for NGF mRNA, followed by that for GDNF mRNA. The NGF mRNA level started to increase 1 hr after the transection, peaked at 6 or 12 hr, and gradually declined until 48 hr. The GDNF mRNA started to rise slightly later, at 3 hr after the injury, attained its maximal level 12 hr postoperation, and gradually declined until 48 hr. However, the change in the mRNA level of BDNF was relatively small, though significant; i.e., an increase was detected first at 6 hr and was sustained until 24 hr after the injury. These results for NGF and GDNF gene expression are in agreement with previous findings (Widenfalk et al., 2001; Murakami et al., 2002). We were particularly interested in GDNF with respect to spinal cord regeneration, because GDNF has potent neurotrophic activity on motoneurons and CNS neurons that send axons into the spinal cord.

Injury-Induced mRNA Expression of Chemokines

Various chemokines are up-regulated in the injured CNS, including the spinal cord (McTigue et al., 1998; Huang et al., 2000; Lee et al., 2000). They are thought to act as chemoattractants to recruit immune cells, such as leukocytes and microglia/macrophages, to the injury site, thus triggering inflammatory reactions. To compare the gene expression profile of GDNF with that of various chemokines, we monitored by RT-PCR the time-dependent mRNA expression of MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and regulated upon activation normal T-cell expressed and secreted (RANTES) in the segment adjacent to the transection site of the spinal cord. The expression profiles of these mRNAs were similar to each other. Especially, the pattern of MIP-1β quite resembled that of MIP-1α, so the results for MIP-1β are not shown. The expression of all chemokines tested was evoked 1 hr after the injury, peaked at 6 or 12 hr, and was sustained at a significantly high level until 48 hr (Fig. 2). These patterns resembled the expression profile of GDNF mRNA, suggesting shared mechanisms for expression between chemokines and GDNF in the injured spinal cord.

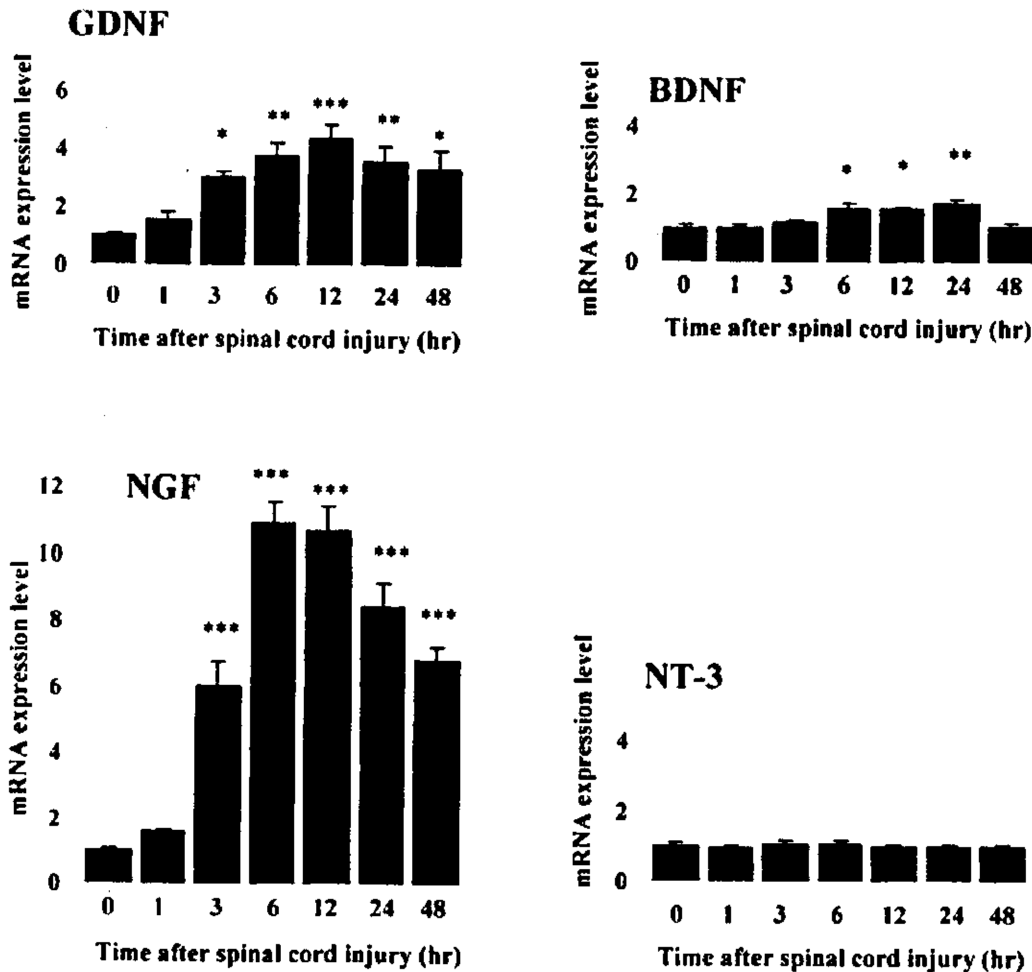


Fig. 1. Expression of neurotrophic factor mRNAs in the sites adjacent rostrally or caudally to the transection site of the rat spinal cord. The time-dependent mRNA expression of neurotrophic factors, including GDNF, NGF, BDNF, and NT-3, after spinal cord transection was monitored by RT-PCR. Total RNA of the tissue was subjected to RT-PCR using primers specific for the respective target genes (see Table I), and PCR products were applied to 2% agarose gels. The bands corresponding to neurotrophic factor mRNA- or β-actin mRNA-derived cDNA were densitometrically quantified, and the ratio of the intensity of each neurotrophic factor band to that of the β-actin band (ratio of neurotrophic factor/β-actin) was calculated. The values are expressed as the means ± SE (n = 3) of the -fold increase in the ratio of GDNF/β-actin, regarding the value of the mice of control group (0 hr) as 1.0. Significant differences from the value of the corresponding control group were determined by one-way ANOVA and Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Expression of GDNF and MCP-1 in the Injured Spinal Cord

Mac1/CD11b-positive cells, implying activated microglia/macrophages (Lu and Pitha, 2001; Babcock et al., 2003), appeared in a limited fashion in both rostral and caudal areas just adjacent to the injury site (data not shown). GDNF or MCP-1 immunoreactivity (-ir) was observed predominantly around the injury site and was colocalized with most of the Mac1/CD11b-positive cells (Fig. 3A–F). These results demonstrate that activated microglia/macrophages synthesized both GDNF and MCP-1 in the transected spinal cord. MCP-1-ir (Fig. 3G–I), but not GDNF-ir (data not shown), was colocalized with GFAP-ir in fibrous structures in the area over 5 mm distant from the injury site, implying the expression of MCP-1 in the processes of astrocytes, as reported previously (Che et al., 2001; Babcock et al., 2003). This finding may suggest that the physiological roles of GDNF are at least partially different from those of MCP-1, because glial production of MCP-1 was shown to be important for leukocyte responses to CNS axonal injury (Babcock et al., 2003). The MCP-1-ir-positive and GFAP-ir-negative cells were sparse in the same area distant from the injury site (Fig. 3I). They might resemble neurons morphologically, but they were likely to be activated microglia/

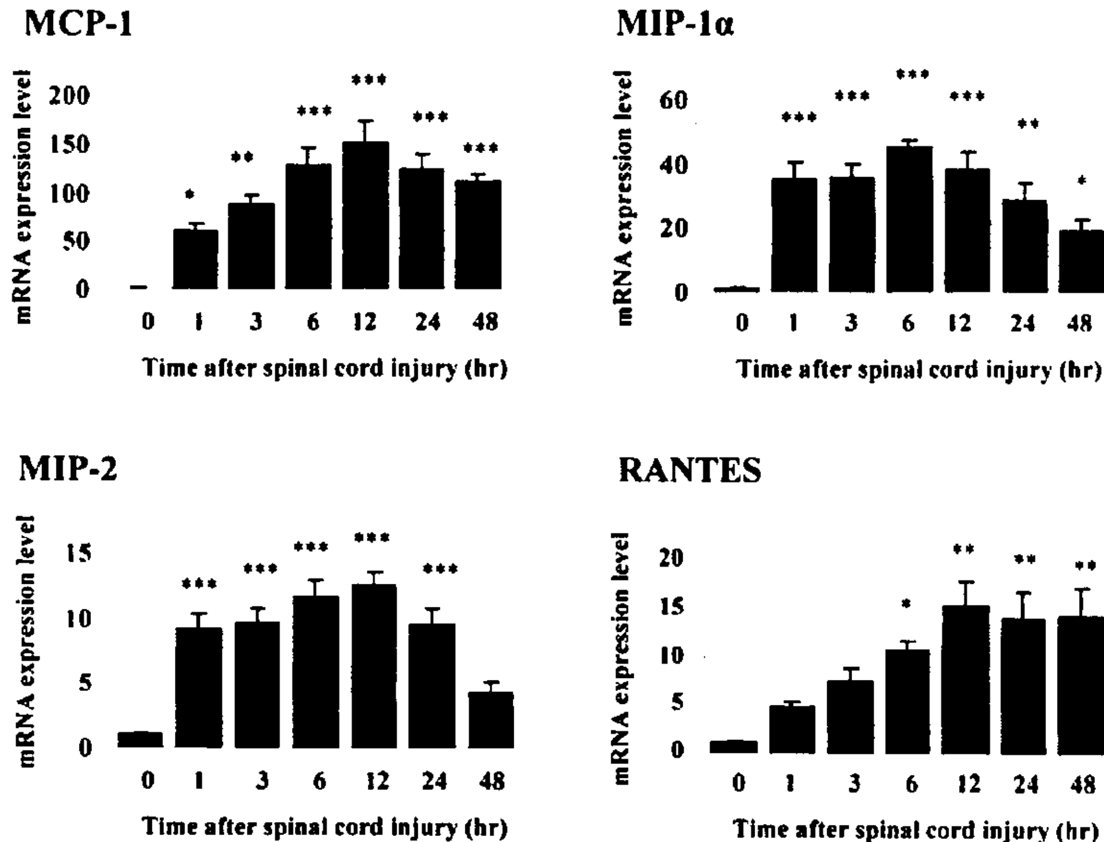


Fig. 2. Expression of chemokine mRNAs in the sites adjacent rostrally or caudally to the transected site of the rat spinal cord. The time-dependent mRNA expression of chemokines, including MCP-1, MIP-1α, MIP-2, and RANTES, after spinal cord transection was monitored by RT-PCR. The ratio of the intensity of chemokine cDNA products to that of β-actin cDNA products was calculated. The values are expressed as the means ± SE (n = 3) of -fold increase over the control group (0 hr). Significant differences from the value of the corresponding control group were determined by one-way ANOVA and Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

macrophages because of intensive MCP-1 expression, which is poor in neurons. Because MCP-1 expression has been shown to occur on injured neurons (Flugel et al., 2001), combined expression of MCP-1 and NeuN, an antigen specific for differentiated neurons, was examined in the areas just adjacent to the injury site. As demonstrated in Figure 3J–L, NeuN and MCP-1 were separately expressed, demonstrating that neurons in the injured spinal cord did not always express MCP-1.

Expression of Receptors for GDNF or MCP-1

To examine whether the activated microglia/macrophages can respond to GDNF, we analyzed by RT-PCR the mRNA expression of receptors for GDNF in macrophages cultured from the rat peritoneal cavity. In addition to the mRNA of MCP-1 receptor CCR2, that of GFRα-1, a glycosyl-phosphatidylinositol (GPI)-anchored binding site of GDNF, was detected, but not that of the tyrosine kinase c-Ret, which transduces the GDNF signal intracellularly (Fig. 4). Recent experiments showed that GDNF actions could be mediated by GFRα-1 in the absence of c-Ret (Paratcha et al., 2003). Therefore, the cultured macrophages used in this work hereinafter seem to respond to GDNF in an autocrine and/or local paracrine manner through GFRα-1 alone.

Effects of GDNF and MCP-1 on Phagocytosis Activity of Cultured Macrophages

Next, dose- and time-dependent effects of GDNF on the phagocytic activity of cultured rat peritoneal macrophages were examined (Fig. 5). Although relatively higher concentrations (100–1,000 ng/ml) and longer exposure times (over 12 hr) were necessary, GDNF significantly facilitated the phagocytosis of latex beads. On the other hand, MCP-1 was effective at a concentrarion as low as 10 ng/ml and showed potent activity as early as 1 hr after exposure. LPS caused a high level of phagocytic activity at 100 ng/ml. The GDNF activity was completely lost when the macrophages were pretreated with PI-PLC, which liberates GFRα-1, the GPI-anchored specific binding site of GDNF, demonstrating a specific and direct action of GDNF on macrophage phagocytosis via this

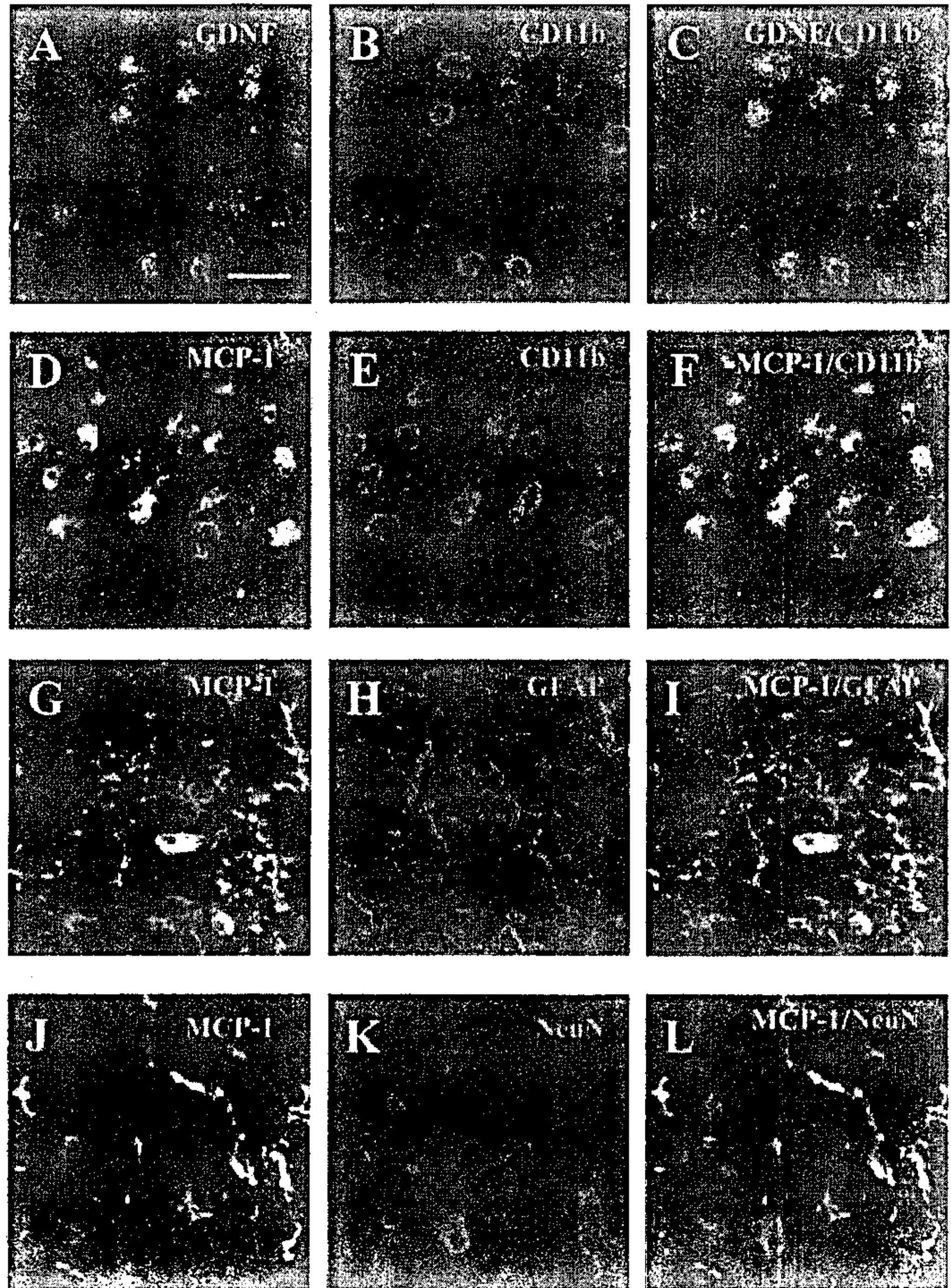


Fig. 3. Localization of GDNF- and MCP-1-ir in the injured rat spinal cord. Rats were anesthetized and cardioperfused 2 days after spinal cord transection. The spinal cord was dissected out, postfixed, and frozen in embedding compound. Coronal sections of 30 μm thickness were cut from the area 2 mm (**A–F**) or 5 mm (**G–L**) distant from the transection site of the spinal cord and used for immunostaining analysis. The sections were reacted with specific antibody against GDNF (A), MCP-1 (D,G,J), GFAP (H), NeuN(K) or CD11b (B,E) and visualized with FITC-conjugated anti-rabbit IgG (A,D,G,J) or rhodamine-conjugated anti-mouse IgG (B,E,H,K). A and B are merged in C, D and E in F,G and H in I, and J and K in L. Scale bar = 20 μm.

GPI-linked receptor component. On the other hand, MCP-1 was fully active, but LPS showed only half of its original activity toward the PI-PLC-treated macrophages. A part of the phagocytic activity elicited by LPS was shown to be mediated through GPI-anchored CD14 (Schiff et al., 1997). Therefore, the PI-PLC treatment liberated not only GFRα-1 but also CD-14 from the surface of the macrophages, resulting in the reduction of GDNF or LPS activity. Neither GDNF nor MCP-1, nor their combination, showed any mitotic activity on macrophages, as judged from the lack of increase in cell number (data not shown). These results demonstrate that GDNF has phagocytosis-stimulating activity, like MCP-1 and LPS, and suggest that GDNF behaves as a cytokine

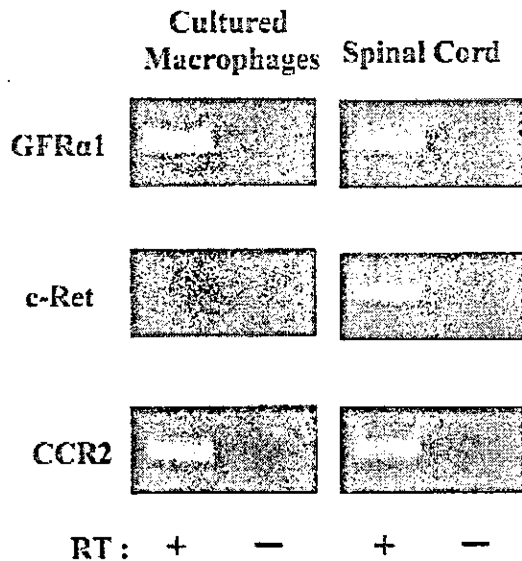


Fig. 4. Expression of GDNF and MCP-1 receptors in rat peritoneal macrophages. RT-PCR analysis detected mRNAs of GFRα-1 and CCR2 in total RNA prepared from cultured rat peritoneal macrophages but no c-Ret mRNA (RT^{+}). No signal was obtained when the RT reaction was conducted without reverse transcriptase (RT−).

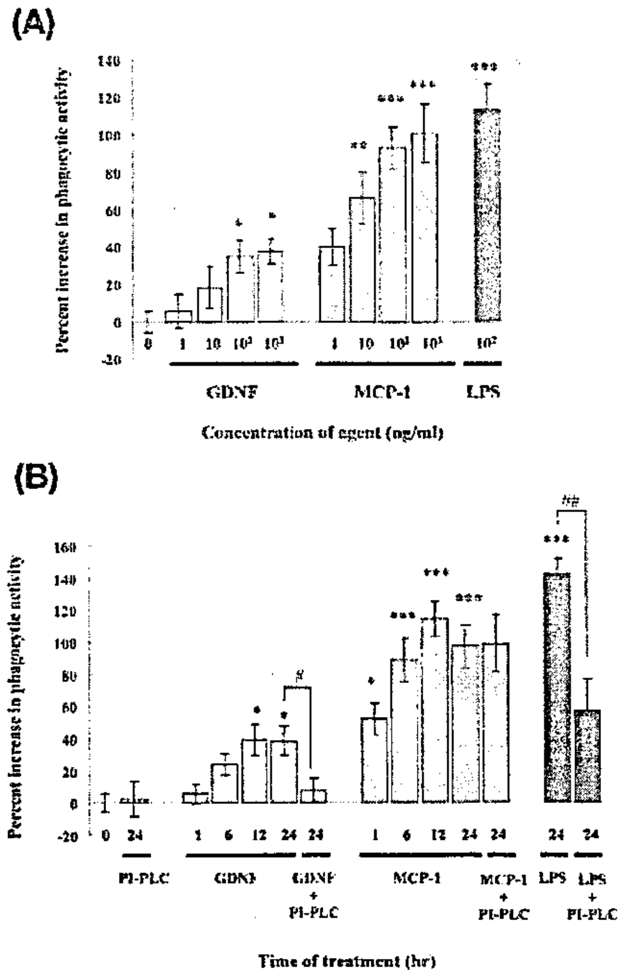


Fig. 5. Dose-dependent (A) and time-dependent (B) increase in phagocytic activity of cultured macrophages after exposure to GDNF, MCP-1, or LPS. Peritoneal macrophages were cultured in the presence of FITC-labeled latex beads after incubation with or without GDNF, MCP-1, or LPS at the indicated concentrations for 24 hr (A) or at 100 ng/ml for the indicated times (B). Some of the cells were pretreated with PI-PLC (0.5 U/ml) for 1 hr to liberate GPI-anchored binding sites, such as GFRα-1 for GDNF and CD14 for LPS. Phagocytic activity is expressed as percentage increase in the number of macrophages bearing FITC-labeled beads/total number of macrophages (mean ± SE, n = 5). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the value of the control group (0 hr, without any reagents; one-way ANOVA and Tukey test); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the value of the group treated with PI-PLC (Student's *t*-test).

that acts on microglia/macrophages in the injured spinal cord.

Effects of GDNF on mRNA Expression of MCP-1 in Cultured Macrophages

Next, the effects of GDNF on the mRNA expression of MCP-1 and its own mRNA were investigated. As shown in Figure 6, GDNF stimulated significantly the expression of MCP-1 mRNA at 100 and 1,000 ng/ml but did not induce any significant elevation of GDNF mRNA. MCP-1 markedly stimulated the expression of MCP-1 mRNA as low as 10 ng/ml, but that of GDNF mRNA only at higher concentrations 100 and 1,000 ng/ml. LPS stimulated both mRNAs at a concentration of 100 ng/ml. These results suggest that the phagocytosis-stimulating activity of GDNF is mediated by GDNF-induced MCP-1.

Activation of the Macrophages Cultured From GDNF Gene Mutants

To clarify the involvement of GDNF in the activation processes of macrophages, we tested the effects of agents that activate macrophages on the macrophages cultured from GDNF gene-deficient +/− or +/+ mice. Macrophages from homozygous mice (−/−) could not be used for this experiment, because the mice died immediately after birth. First, mRNA expression of GDNF, GFRα-1, and c-Ret gene in the cerebral cortex and cultured macrophages was analyzed by RT-PCR (Fig. 7A). GDNF, GFRα-1, and c-Ret mRNAs were expressed in the cerebral cortex of both mutant (+/−) and +/+ littermates. However, c-Ret mRNA was not expressed in the macrophages of either genetic type, consistent with the data on cultured rat macrophages shown in Figure 4. The expression level of GDNF mRNA was always lower in the +/− than in the +/+ mice before and after the stimulation with MCP-1 or LPS (Fig. 7A,Ba). The level of GFRα-1 mRNA tended to be lower in the +/− than in the +/+ mice in both cerebral cortex and

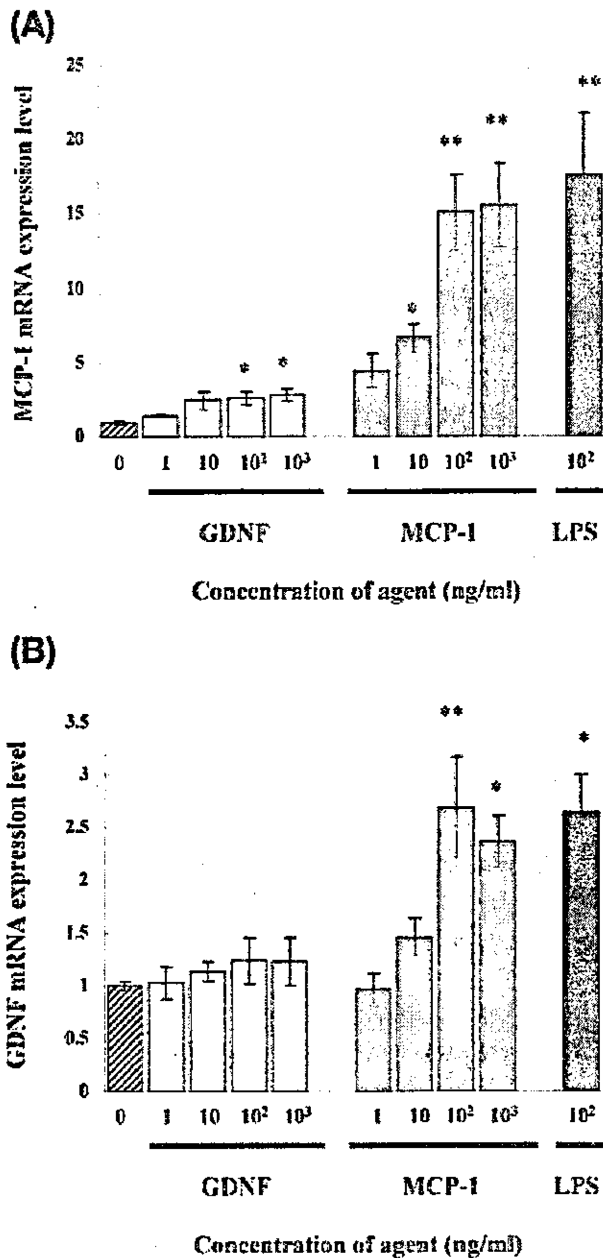


Fig. 6. Dose-dependent effects of GDNF, MCP-1, and LPS on the mRNA expression of MCP-1 (A) or GDNF (B) of cultured rat peritoneal macrophages. Macrophages were cultured with or without MCP-1, GDNF, or LPS at various concentrations for 24 hr, and then total RNA of the macrophages was prepared and subjected to RT-PCR with primers specific for MCP-1 (A) or GDNF (B). The ratio of the intensity of cDNA products to that of β-actin cDNA products was calculated. The values are expressed as mean ± SE (n = 3) of -fold increase over the control group (0 ng/ml). Significant differences from the value of the control group (0 ng/ml) were determined by one-way ANOVA and Tukey post hoc test. $*P < 0.05$, $**P < 0.01$.

macrophages (Fig. 7A), but the difference was not statistically significant (Fig. 7Bb). MCP-1 mRNA expression was markedly facilitated by the stimulation with MCP-1 or LPS but was the same for both genetic types (Fig. 7Bc).

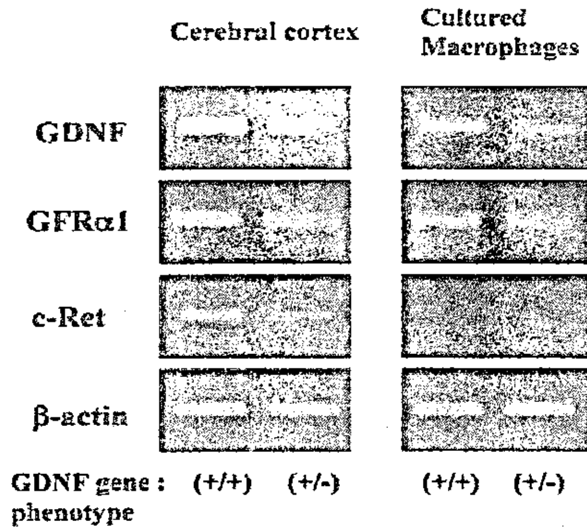
There were no morphological differences in the macrophages with respect to the genetic type or stimulant (Fig. 8A). The phagocytosis activity of the +/+ macrophages was significantly enhanced by the treatment with GDNF, MCP-1, or LPS or with MCP-1 or LPS but not with GDNF, in the +/− cells (Fig. 8B). However, the value of the phagocytosis activity induced was significantly lower in the +/− than in the +/+ macrophages when the cells were treated with GDNF, MCP-1, or LPS. The data thus indicate that the GDNF signal is important and is involved in phagocytosis-stimulating processes, i.e., activation processes of macrophages.

DISCUSSION

Our present study was aimed at addressing the physiological significance of GDNF produced by macrophages in the injured CNS, and the results suggest that GDNF plays a role as a cytokine to activate microglia/macrophages in an autocrine/paracrine manner. Our observation that mRNAs of GDNF, NGF, BDNF, and all chemokines examined were up-regulated around the injury site of the rat spinal cord is essentially consistent with previous findings (McTigue et al., 1998; Lee et al., 2000; Satake et al., 2000; Widenfalk et al., 2001). However, detailed expression profiles of these factors had not been studied before. The mRNA expression profile of GDNF resembled very much that of NGF or various chemokines, including MCP-1 (Figs. 1, 2). Indeed, NGF is a well-known cytokine that stimulates phagocytosis and chemotaxis of macrophages (Susaki et al., 1996; Kobayashi and Mizisin, 2001). These findings prompted us to examine whether GDNF plays an inflammatory role following spinal cord injury. We focused on GDNF and compared its biological actions particularly on cultured macrophages with those of MCP-1, because GDNF and MCP-1 were coexpressed in microglia/macrophages around the transected site of the spinal cord (Fig. 3). Furthermore, Batchelor et al. (1999) reported that the synthesis of GDNF was increased in macrophages around the site of striatal injury, demonstrating a possibility that GDNF participates as a cytokine or chemokine to induce inflammatory reactions following spinal cord injury.

We initially examined the expression of the receptors of GDNF or MCP-1 in rat peritoneal macrophages, and we detected GFRα-1 mRNA, but not c-Ret mRNA (Figs. 4, 7). Generally, GDNF first binds to GFRα-1, and this complex then activates Ras/ERK and phosphatidylinositol 3-kinase/Akt pathways through the tyrosine kinase receptor c-Ret (Jing et al., 1996; Trupp et al., 1999). However, GDNF can also signal through activation of Src family kinase via GFRα-1 in the absence of c-Ret (Trupp et al., 1999; Pezeshki et al., 2001). In fact, exogenous GDNF enhanced phagocytic activity of the cultured macrophages via GFRα-1, a GPI-anchored GDNF binding protein (Fig. 5), demonstrating that GDNF could directly

A



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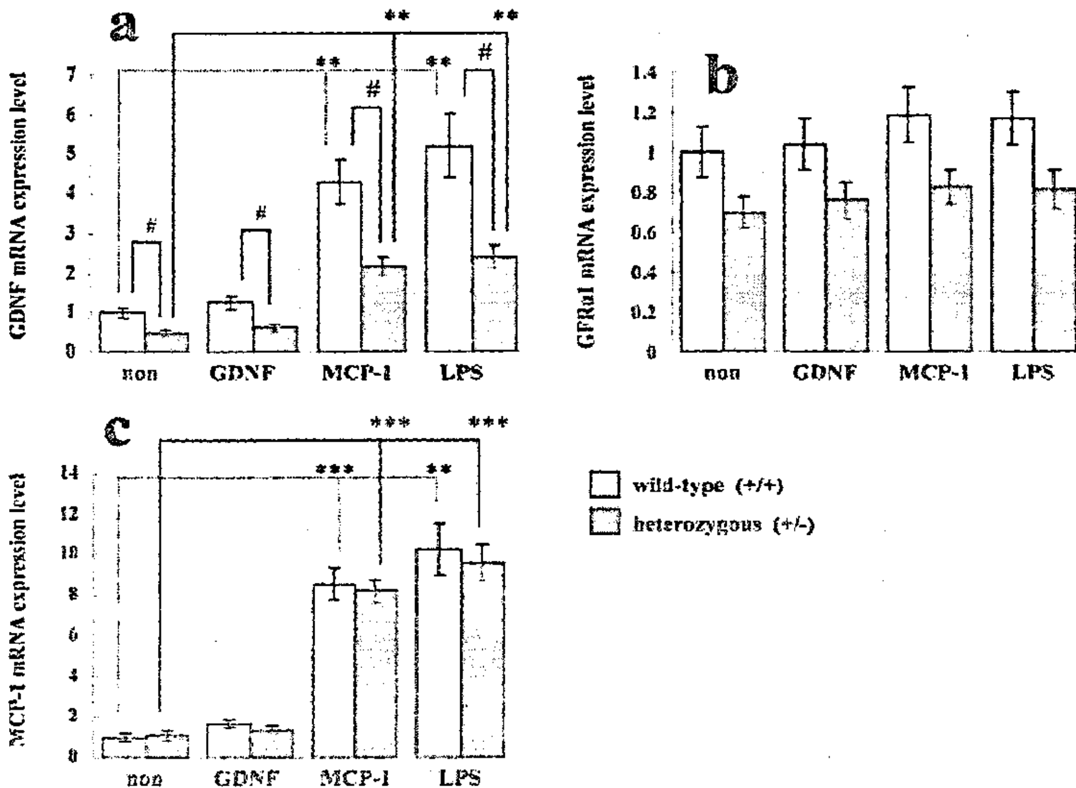

Fig. 7. Effects of GDNF, MCP-1, and LPS on mRNA expression of GDNF and its receptors, GFRα-1 and c-Ret, in the macrophages cultured from wild-type mice or GDNF gene mutants. **A:** mRNA expression of GDNF and of its receptors, GFRα-1 and c-Ret, in the cerebral cortex and cultured macrophages of GDNF gene-deficient heterozygous mice (+/−) and wild-type mice (+/+) was examined by RT-PCR. The macrophages were cultured with or without GDNF, MCP-1, or LPS (100 ng/ml) for 24 hr, after which total RNA of the cells was prepared and subjected to RT-PCR with primers specific for GDNF, GFRα-1, or MCP-1. **B:** The ratio of the intensity of cDNA products to that of the β-actin cDNA product was calculated. The values are expressed as mean ± SE (n = 3) of -fold increase over the nontreatment group of +/+ macrophages. Statistical significance was assessed using one-way ANOVA followed by Tukey post hoc test. Significance of differences as indicated by the brackets: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$.

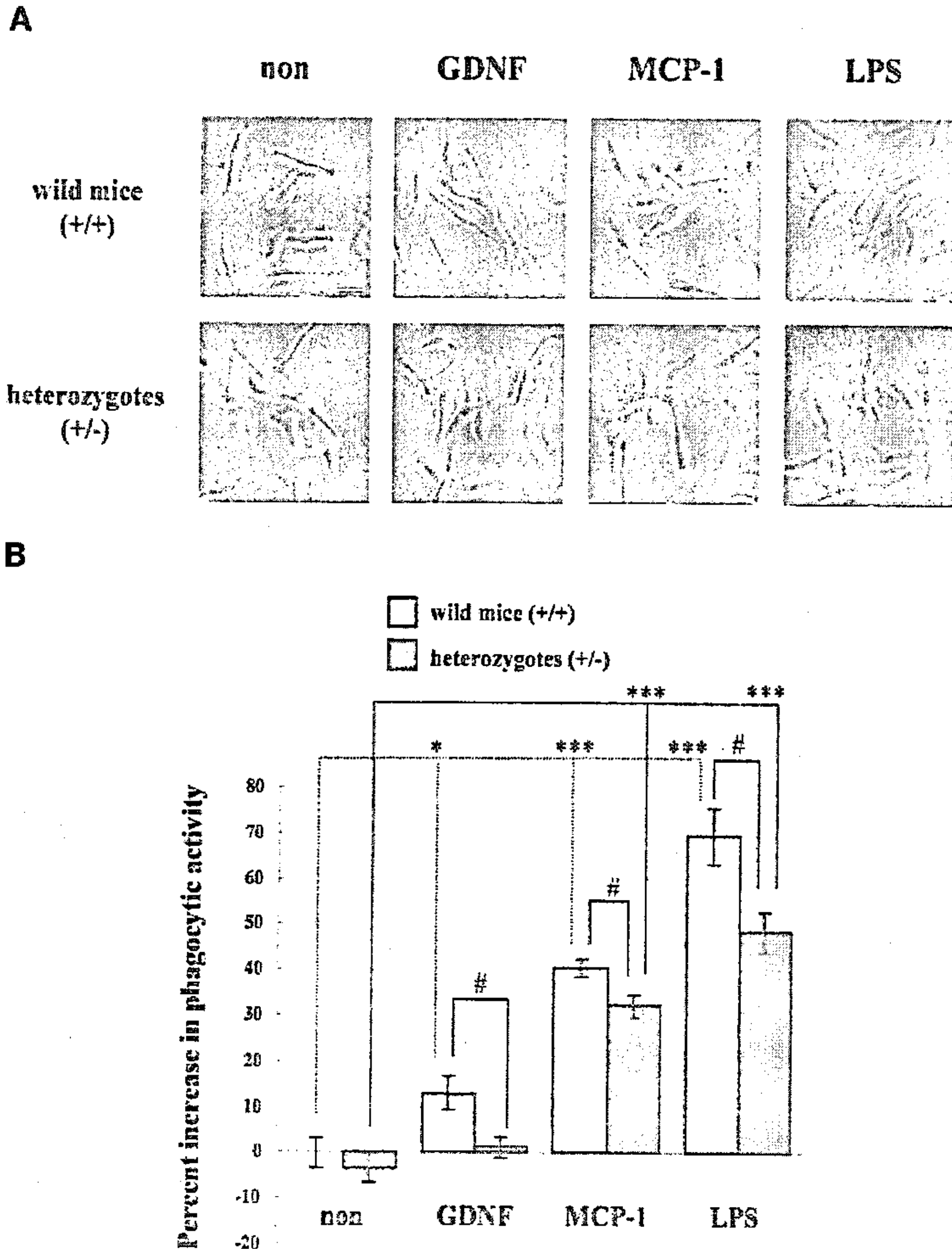

Fig. 8. Effects of GDNF, MCP-1, and LPS on the morphology (A) and phagocytic activity (B) of the macrophages cultured from wild-type mice or GDNF gene mutants. A: Macrophages from wild-type mice (+/+) or GDNF gene-deficient heterozygous mice (+/–) were cultured for 24 hr in medium supplemented or not with GDNF, MCP-1, or LPS (100 ng/ml), and then photographs of them were taken under a phase-contrast microscope. B: The +/+ or +/– macrophages were cultured for 30 min in medium containing FITC-labeled latex beads after incubation with or without 100 ng/ml of GDNF, MCP-1, or LPS for 24 hr. FITC-positive cells (phagocytosing macrophages) were counted under a fluorescence microscope, and the percentage of positive cells per total cells was calculated. The values are expressed as mean ± SE (n = 4) of the percentage increase over the control group (non). Statistical significance was assessed using one-way ANOVA followed by Tukey post hoc test. Significance of differences as indicated by the brackets: $^{*}P < 0.05$, $^{***}P < 0.001$, $^{\#}P < 0.05$. Scale bar = 20 μm.

influence macrophage function through activation of Src family kinase in a c-Ret-independent manner. This implies an autocrine/paracrine action of GDNF on macrophages.

It is likely that GDNF stimulated phagocytosis of macrophages by the mediation of GDNF-induced chemokines, such as MCP-1. However, MCP-1 mRNA was expressed at a similar level in both +/+ and mutant +/− macrophages before or after stimulation (Fig. 7Bc) in spite of the lower expression of GDNF mRNA in the mutant macrophages (Fig. 7Ba). Therefore, GDNF mRNA expression is correlated better than MCP-1 mRNA expression with induction of the phagocytic activity, which may be supported by our preliminary result showing that the mutant macrophages secreted about half the GDNF protein secreted by the wild-type mice. It is possible that GDNF directly participates in the activation processes of the macrophages.

Exogenously infused GDNF can exert behavioral and anatomic neuroprotection against spinal cord injury (Cheng et al., 2002). Therefore, GDNF produced after a spinal cord injury is likely to exert predominantly neurotrophic effects on injured neurons. It is also conceivable that GDNF produced from microglia/macrophages in the injured spinal cord induces more activation of macrophages and more expression of GDNF and MCP-1 from these activated macrophage and that the increased GDNF acts as a neurotrophic factor for injured neurons. However, successful regeneration after a spinal cord injury is difficult, because the inflammatory response, such as activation of macrophages, is delayed and limited. In fact, GDNF might not have been produced so much because of the limited number of activated macrophages. Zeev-Brann et al. (1998) showed that activation of microglia/macrophages was enhanced when the cells were exposed to sciatic nerve segments but was inhibited in the presence of optic nerve segments, suggesting the existence of substances in the CNS inhibitory toward the activation of macrophages. Such inhibition may reduce the neurotrophic influence of GDNF in the injured site and result in failed regeneration. Macrophages are also known to have not only beneficial but also harmful effects, the latter caused by the production of nitric oxide. Indeed, infusion of a chemokine antagonist attenuated macrophage infiltration and induced axonal sprouting in the injured spinal cord (Ghirnikar et al., 2001). This is contradictory to the beneficial effects of macrophages. We propose that a delayed inflammatory response may have a harmful influence on a spinal cord injury but that an early one may be beneficial for neuronal regeneration because of an increased production of neurotrophic factors such as GDNF (Fig. 1).

In summary, we demonstrate here that GDNF was up-regulated in microglia/macrophages after spinal cord transection and enhanced phagocytic activity and expression of GDNF or chemokine mRNA of cultured rodent peritoneal macrophages. GDNF produced after a spinal cord injury might not only have a beneficial influence on the injury but might also participate in the inflammatory response.

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Role of tumor necrosis factor-α in down-regulation of hepatic cytochrome *P*450 and P-glycoprotein by endotoxin

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Abstract

We investigated the role of tumor necrosis factor-α (TNF-α) in the down-regulation of hepatic P-glycoprotein and cytochrome *P*450 (CYP) by endotoxin, using TNF-α gene-deficient (TNF-$\alpha^{-/-}$) mice. In the case of P-glycoprotein, endotoxin (10 mg/kg) significantly decreased the expression of hepatic P-glycoprotein in wild-type mice 6 h, but not 24 h, after intraperitoneal injection, with no significant differences in the constitutional expression of P-glycoprotein between wild-type mice and TNF-$\alpha^{-/-}$ mice. However, endotoxin had no effect on the expression of P-glycoprotein in TNF-$\alpha^{-/-}$ mice either 6 or 24 h after injection. When doxorubicin was administered intravenously to TNF-$\alpha^{-/-}$ mice treated 6 h earlier with and without endotoxin, no significant differences in the plasma concentrations of doxorubicin 3 h after injection were observed between endotoxin-treated and untreated TNF-$\alpha^{-/-}$ mice. These results suggest that TNF-α plays a pivotal role in the down-regulation of P-glycoprotein by endotoxin. In the case of CYP, the constitutive expression of hepatic CYP3A2 and CYP2C11 had a tendency to decline in TNF-$\alpha^{-/-}$ mice compared with that in wild-type mice. Endotoxin significantly decreased the expression of hepatic CYP3A2 and CYP2C11 in wild-type mice 24 h after injection, and that decreased expression was significantly greater in TNF-$\alpha^{-/-}$ mice than wild-type mice. When antipyrine was administered intravenously to wild-type mice and TNF-$\alpha^{-/-}$ mice treated 24 h earlier with endotoxin, the plasma concentrations of antipyrine in TNF-$\alpha^{-/-}$ mice 3 h after injection were significantly higher than those in wild-type mice. These findings suggest that TNF-α plays a key role in endotoxin-induced down-regulation of hepatic P-glycoprotein, as well as plays a protective role in the regulation of hepatic CYP3A2 and CYP2C11 against endotoxin-induced acute inflammatory response. In TNF-$\alpha^{-/-}$ mice, other cytokines appear to function as compensation for the lack of endogenous TNF-α.
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Keywords: Tumor necrosis factor-α (TNF-α); Cytochrome *P*450 (CYP); P-glycoprotein; Endotoxin; TNF-α knockout mice

1. Introduction

It is well known that bacterial infections impair hepatic drug metabolism in humans, and that endotoxin (lipopolysaccharide), a major component of the cell wall of Gram-negative bacteria, plays a key role in this phenomenon. Endotoxin is known to secrete various inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-2, and interleukin-6. These inflammatory mediators have been shown to regulate the content and activities of hepatic cytochrome *P*450 (CYP) in humans and animals (Chen et al., 1992; Manuel, 1996; Morgan, 1997; Shedlofsky et al., 1994). Among them, NO is significantly released after exposure to endotoxin, subsequent to the expression of inducible NO synthase (Bredt and Snyder, 1994; Khatsenko et al., 1993; Moncada et al., 1991). We and other investigators have demonstrated that NO is one

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of the important inflammatory mediators regulating the contents and activities of CYP (Kitaichi et al., 1999, 2004; Gergel et al., 1997; Khatsenko and Kikkawa, 1997; Khatsenko et al., 1993; Minamiyama et al., 1997; Morgan, 1997; Sewer and Morgan, 1997; Takemura et al., 1999; Ueyama et al., 2004; Wink et al., 1993). On the other hand, it has also been reported that the endotoxin-induced down-regulation of CYP3A2, CYP2C11, and CYP2E1 is NO-independent (Sewer and Morgan, 1997, 1998). The mechanism responsible for the NO-mediated down-regulation of CYP isoforms is still unclear.

In addition to NO, TNF-α is also thought to be of major importance in the down-regulation of CYP isoforms in endotoxemia. It has been reported that TNF-α decreases the contents of the CYP isoforms CYP3A2 and CYP2C11 in rats (Monshouwer et al., 1996; Sewer and Morgan, 1997). In contrast, Warren et al. (1999), in animal experiments using TNF-α receptor (p55/p75)-deficient mice, reported that TNF-α does not play a key role in the endotoxin-induced down-regulation of CYP isoforms, including CYP1A and CYP3A.

On the other hand, inflammatory cytokines, including TNF-α, interleukin-1, interleukin-2, and interleukin-6, might play an important role in endotoxin-induced changes in certain drug transporter-mediated hepatobiliary excretion systems (Hirsch-Ernst et al., 1998; Simpson et al., 1997). We previously reported that *Klebsiella pneumoniae* endotoxin significantly reduces hepatobiliary excretion of the β-lactam antibiotic, cefoperazone, which is a substrate for multidrug resistance-associated protein 2 (Mrp2) (Haghgoo et al., 1995; Nadai et al., 1998), suggesting that some inflammatory mediators released by endotoxin contribute to the impairment of the hepatobiliary excretion of drugs by reducing the expression and/or function of Mrp2 in the canalicular membrane of hepatocytes. Endotoxin is known to induce cholestasis and hyperbilirubinemia by down-regulating Mrp2, an efflux pump for bile acids and bilirubin, due to the secretion of some cytokines, including TNF-α and interleukin-1 (Green et al., 1996; Nakamura et al., 1999; Trauner et al., 1997). In contrast, there is evidence that TNF-α induces the up-regulation of transporter genes or MRP1 protein in human colon carcinoma cells and mdr1 in rat hepatoma cells (Chapekar et al., 1991; Stein et al., 1997). However, the precise role of TNF-α in the down-regulation or up-regulation of hepatic Mrp2 by endotoxins remains unclear.

Like Mrp2, the ATP-binding cassette transport protein, P-glycoprotein, is expressed in many eliminating organs such as the liver and kidney (Cordon-Cardo et al., 1989; Thiebaut et al., 1987), and acts as the efflux transport protein for endogenous and exogenous toxic substances (Schinkel et al., 1996; Thiebaut et al., 1987). Thus, these two drug transport proteins, P-glycoprotein and Mrp2, might exert a protective function of excluding various lipophilic substrates from the liver. There is evidence that TNF-α reduces the expression of P-glycoprotein (Sukhai et al., 2000). In our previous studies, it was suggested that endotoxin dramatically decreases the P-glycoprotein-mediated hepatobiliary excretion of rhodamine 123 by reducing the expression of mdr1a due to increased plasma TNF-α levels (Ando et al., 2001). From the above findings, the expression of both hepatic P-glycoprotein and Mrp2 appears to be regulated by inflammatory cytokines, including TNF-α. Interestingly, there is evidence that the numerous substrates of P-glycoprotein, CYP3A, and Mrp2 largely overlap, and that these proteins are located at hepatocytes and have similar functions of removing various drugs from the body (Mayer et al., 1995; Oude Elferink et al., 1995; Wacher et al., 1995). On the basis of these observations, it is possible that endotoxin might simultaneously down-regulate hepatic P-glycoprotein, CYP3A, and Mrp2. However, to our knowledge, there is no information confirming whether endotoxin simultaneously regulates the expression of CYP3A, P-glycoprotein, and Mrp2. Taken together, it is, at present, difficult to clarify the role of endogenous TNF-α in the regulation of hepatic CYP isoforms and drug transporters.

In the present study, we focused on the effect of TNF-α on the expression of the drug transporter P-glycoprotein and the major CYP isoforms, CYP3A2 and CYP2C11. It is considered that mice with targeted deletions of the TNF-α gene (TNF-$\alpha^{-/-}$ mice) are useful as an animal model for evaluating the role of endogenous TNF-α in the down-regulation of CYP isoforms by endotoxin. The aim of the present study was to clarify the role of TNF-α in the endotoxin-induced down-regulation of P-glycoprotein and these CYP isoforms, using mice with a targeted disruption of the gene encoding TNF-α (Taniguchi et al., 1997), which can block the effects of TNF-α.

2. Materials and methods

2.1. Chemicals

Endotoxin was obtained from *K. pneumoniae* LEN-1 (O3:K1$^-$), which was identical to that used in previous studies (Ando et al., 2001; Kitaichi et al., 1999; Nadai et al., 1998; Zhao et al., 2002). Doxorubicin hydrochloride and daunorubicin hydrochloride were purchased from Sigma (St. Louis, MO, USA). Doxorubicin hydrochloride, in the form of a commercial preparation for injection, was purchased from Kyowa Hakko Kogyo (Adriamycin; Tokyo, Japan). Antipyrine, testosterone, 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 2α-hydroxytestosterone were purchased from Sigma. All other chemicals were commercially available and were of analytical grade. Endotoxin and antipyrine were dissolved in isotonic saline.

2.2. Animals

Nine- to 10n-week-old male C57BL/6$^-$ TNF-$\alpha^{-/-}$ and C57BL/6 mice were used in the present experiment.

C57BL/6^{-} TNF-$\alpha^{-/-}$ mice were obtained from embryonic stem (ES) cells of the TT2 line by backcrossing C57BL/6 by more than eight generations (Taniguchi et al., 1997). The wild-type mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed under controlled environmental conditions (temperature of 22–24 °C and humidity of 55±5%) with a commercial food diet and water freely available to the animals. All animal experiments were carried out in accordance with the guidelines of the Nagoya University School of Medicine for the care and use of laboratory animals.

2.3. Histopathological examinations

Mice under light anesthesia with diethyl ether were killed by exsanguination 24 h after injection of endotoxin or saline. For light microscopy, the liver was immediately removed and small pieces of liver tissues were fixed in 20% formaldehyde in 0.1 M phosphate-buffered saline (PBS). Fixed tissue specimens were embedded in paraffin wax, and paraffin sections were then treated with hematoxylin and eosin stain. Veterinary pathologists performed the histopathological examinations according to a method reported previously (Zhao et al., 2002).

2.4. Elimination of antipyrine and doxorubicin

Under light anesthesia with sodium pentobarbital, the right jugular vein of each mouse was cannulated with a polyethylene tube for the injection of antipyrine or doxorubicin. Doxorubicin (30 mg/kg) was administered intravenously in TNF-$\alpha^{-/-}$ mice 6 h after injection of endotoxin (10 mg/kg) or saline. Antipyrine (20 mg/kg) was administered intravenously in wild-type and TNF-$\alpha^{-/-}$ mice 24 h after injection of endotoxin or saline. Three hours after injection of doxorubicin or antipyrine, blood samples were collected by exsanguinations from the abdominal aortas of mice under light anesthesia with diethyl ether. Plasma samples were obtained from the blood samples by centrifugation at 4000×g for 10 min at 4 °C, and were stored at −40 °C until analyzed.

2.5. Hepatic microsome preparation for Western blot

Mice were anesthesized with sodium pentobarbital (25 mg/kg) 24 h after injection of endotoxin or saline. Then, each liver was excised after perfusion of 10 ml of iced-cold saline to remove most of the blood. The microsomes were prepared according to the method of Omura and Sato (1964). Briefly, the liver (approximately 0.5 g) was homogenized at 4 °C with a tight homogenizer (10 strokes up and down) using 1.15% KCl. The homogenate was centrifuged at 12,000×g for 25 min at 4 °C. The supernatant was further centrifuged at 80,000×g for 90 min at 4 °C to obtain the microsomal fraction. The obtained pellet was washed with 1.15% KCl and then resuspended in 1.15% KCl. The protein concentration of the microsomal fraction was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin (Sigma) as a standard. The fraction was kept at −80 °C until analysis.

2.6. Testosterone hydroxylase activity

The liver (approximately 0.5 g) obtained by the methods described above was homogenized at 4 °C with Teflon homogenizer (10 strokes up and down) using a buffer solution consisting of 250 mM sucrose, 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, and 10 mM KCl. The homogenate was centrifuged at 9000×g for 25 min at 4 °C. The supernatant was further centrifuged at 105,000×g for 60 min at 4 °C to obtain the microsomal fraction. The obtained pellet was dissolved in the buffer solution, and again centrifuged at 105,000×g for 60 min at 4 °C.

A volume of 480 μl of 100 mM phosphate-buffered incubation medium (pH 7.4) containing 3.3 mM $MgCl_2$, 1.5 mM β-$NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase (G6PD), and 50 μl of the obtained microsomal protein (about 1 mg/ml protein) was placed in a sample tube, and preincubated for 1.5 min at 37 °C. The reaction was initiated by addition of 20 μl of 5 mM testosterone as a substrate. Incubations were performed for 15 min at 37 °C and were terminated by adding 1 ml of ice-cold ethyl acetate and 20 μl of 250 mM 11α-hydroxyprogesterone as internal standard. The samples were vortex-mixed for 30 s and centrifuged at 11,000×g for 10 min. The organic phase was evaporated under a nitrogen stream, and the residue was dissolved in 50% methanol and subjected to high-performance liquid chromatography (HPLC) analysis.

2.7. Western blot analysis for CYP3A2 and CYP2C11 in microsomal fraction

The protein (1 μg) was separated by electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked in PBS solution containing 0.1% Tween 20 and 4% nonfat dry milk, and detected by rabbit polyclonal antibody to rat CYP3A2 (Daiichi Pure Chemicals, Tokyo, Japan) and goat polyclonal antibody to rat CYP2C11 (Daiichi Pure Chemicals). Immune complexes were visualized using horseradish peroxidase-labeled secondary antibody, antirabbit IgG (Amersham Biosciences, Piscataway, NJ, USA) for CYP3A2, and antigoat IgG (Sigma) for CYP2C11 with ECL Western blot detection reagents (Amersham Biosciences).

To quantify the relative levels of each protein in each gel, the intensity of the stained bands was measured by the NIH image program (Bethesda, MD, USA). The levels were expressed as 100% of those in mice treated with saline in place of endotoxin.

2.8. Western blot analysis for hepatic P-glycoprotein

The liver was obtained from mice 24 h after injection of endotoxin or saline. Each liver was excised after a perfusion of 10 ml of iced-cold saline to remove most of the blood. Each liver (approximately 0.2 g) was suspended in 10-fold volumes of 10 mM Tris–HCl buffer (pH 8.0) containing 1.5 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (Sigma). The suspension was homogenized with a tight homogenizer (20 strokes up and down) and centrifuged at 3000×g for 10 min at 4 °C. The supernatant was centrifuged at 30,000×g for 60 min at 4 °C. The pellet was dissolved in 100 μl of 10 mM Tris–HCl buffer (pH 8.0) containing 0.5% Nonidet P40 (Daiichi Pure Chemicals). The protein (40 μg) was separated by electrophoresis on 8% polyacrylamide gels containing 0.1% SDS and transferred to a PVDF membrane (Millipore). The membrane was blocked in PBS containing 0.1% Tween 20 and 5% nonfat dry milk, and detected by C219 mouse monoclonal antibody to P-glycoprotein (DakoCytomation, Glostrup, Denmark).

The intensity of the stained bands was measured as described above. The levels were expressed as 100% of those in mice treated with saline in place of endotoxin.

2.9. Measurement of plasma nitrate/nitrite (NO_x) levels

Blood samples were collected to determine plasma NO_x (nitrate/nitrite) 24 h after the injection of endotoxin. The plasma samples were ultrafiltered (molecular cutoff of 10,000) at 6000×g for 60 min at 4 °C. The concentration of NO_x in the ultrafiltrate was measured by a commercially available kit (Nitrate/Nitrite Colorimetric Assay Kit; Cayman Chemical, Ann Arbor, MI, USA) using a microplate reader (Molecular Devices, Crawley, UK). Nitrite recovery in this assay was over 95%.

2.10. Drug analysis

HPLC analyses were performed using a Shimadzu LC-10A system (Kyoto, Japan) consisting of an LC-10A liquid pump and an auto injector SIL-10Advp, and equipped with a UV–VIS detector (SPD-10 AV) set at wavelength of 254 nm for antipyrine and 247 nm for testosterone metabolites, and a fluorescence detector (RF-10AXL) (excitation, 480 nm; emission, 560 nm) for doxorubicin. The assay conditions were as follows: column, a Cosmocil $5C_{18}$ (Nacalai Tesque, Kyoto, Japan) for antipyrine and doxorubicin, and a Cosmocil 5CN-MS (Tosho, Tokyo, Japan) for testosterone metabolites; mobile phase, 30% methanol in water (vol/vol) for antipyrine, 0.5% phosphoric acid–methanol (40:60, vol/vol) solution for doxorubicin, and water/methanol/acetonitrile (76:22:2, vol/vol) for testosterone metabolites; temperature, 40 °C for antipyrine and testosterone metabolites, and 50 °C for doxorubicin; flow rate, 1.0 ml/min for antipyrine and testosterone metabolites, and 1.2 ml/min for doxorubicin.

2.11. Statistical analysis

Results are expressed as mean±S.E.M. Statistical differences between means were assessed by Student's t test or one-way analysis of variance (ANOVA). When F ratios were significant (P<0.05), Scheffe's post-hoc tests between the groups were done, and P values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of endotoxin on histopathological findings in wild-type and TNF-$\alpha^{-/-}$ mice

Light micrographs obtained by the histopathological examinations revealed that there was no difference in the light microscopy of liver tissues between wild-type mice and TNF-$\alpha^{-/-}$ mice, indicating that the livers of both possess almost the same morphological characteristics. Endotoxin induced only a mild infiltration with no evidence of a massive necrotic or apoptotic area in either the wild-type or TNF-$\alpha^{-/-}$ mice.

3.2. Effect of endotoxin on expression of hepatic P-glycoprotein in wild-type and TNF-$\alpha^{-/-}$ mice

The time-dependent effects of endotoxin on the expression of P-glycoprotein in wild-type mice and TNF-$\alpha^{-/-}$ mice after intraperitoneal injection of endotoxin (10 mg/kg) are illustrated in Fig. 1. The expression of P-glycoprotein in wild-type mice significantly decreased 6 h after the endotoxin injection but returned to the control level by 24 h. The expression of P-glycoprotein did not change in TNF-$\alpha^{-/-}$ mice either at 6 or 24 h after injection with endotoxin. No significant differences in the constitutive expression of P-glycoprotein were observed between wild-type and TNF-$\alpha^{-/-}$ mice. To evaluate in vivo whether the function of P-glycoprotein is maintained

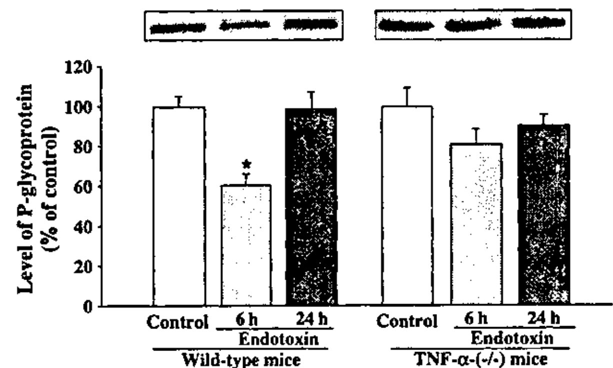

Fig. 1. Time-dependent effects of endotoxin on hepatic expression of P-glycoprotein in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05).

in endotoxin-treated TNF-$\alpha^{-/-}$ mice, we measured the plasma concentrations of doxorubicin 3 h after injection in TNF-$\alpha^{-/-}$ mice treated 6 h earlier with and without endotoxin. No significant differences in the plasma concentrations of doxorubicin were observed between endotoxin-treated and untreated TNF-$\alpha^{-/-}$ mice (0.43±0.02 and 0.34±0.04 μg/ml, respectively), although endotoxin significantly increased the plasma concentrations of doxorubicin in wild-type mice. These results indicate that endogenous TNF-α is involved in the down-regulation of P-glycoprotein.

3.3. *Effect of endotoxin on expression of CYP3A2 and CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice*

The constitutive expression of hepatic CYP3A2 and CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice is represented in Fig. 2. The constitutive expression of CYP3A2 and CYP2C11 in TNF-$\alpha^{-/-}$ mice showed a tendency to decline compared to that in wild-type mice, although the differences failed to reach the 5% level of statistical significance. The effects of endotoxin on the expression of hepatic CYP3A2 and CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice 24 h after injection of endotoxin are illustrated in Figs. 3 and 4. Endotoxin significantly reduced the expression of CYP3A2 and CYP2C11 in both wild-type and TNF-$\alpha^{-/-}$ mice, although the degree of reduced expression in the latter was greater than in the former.

3.4. *Effect of endotoxin on formation from testosterone to its metabolites in wild-type and TNF-$\alpha^{-/-}$ mice*

It is reported that the major metabolites of testosterone, 6β-hydroxytestosterone, and both 16α-hydroxytestosterone and 2α-hydroxytestosterone are indicative of CYP3A2 and CYP2C11 enzymes, respectively (Takahashi et al., 1999).

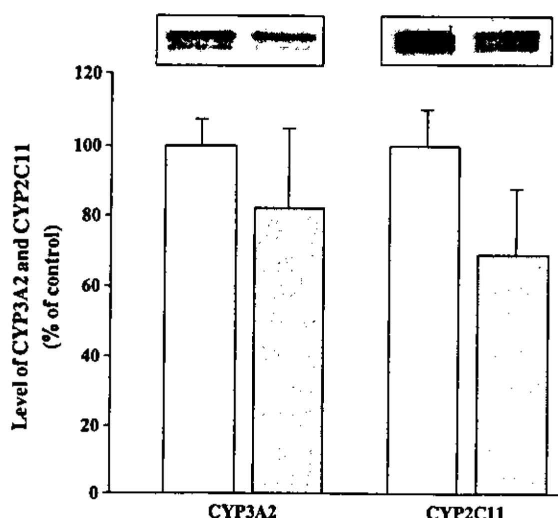

Fig. 2. Constitutive expression of CYP3A2 and CYP2C11 in liver of wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6).

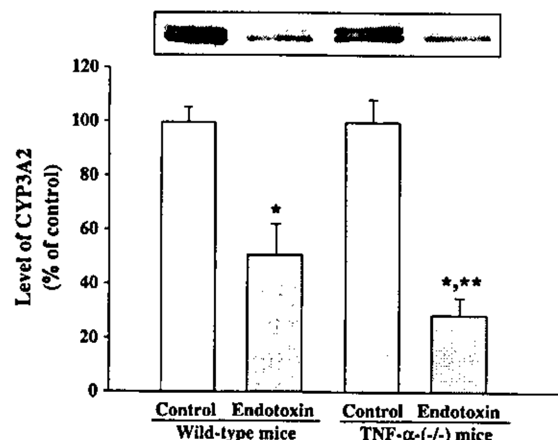

Fig. 3. Effect of endotoxin on hepatic expression of CYP3A2 in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

Therefore, we measured the formation rate of 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 2α-hydroxytestosterone in the microsomal fractions obtained from wild-type and TNF-$\alpha^{-/-}$ mice treated 24 h earlier with or without endotoxin. Endotoxin significantly reduced the formation rate of 6β-hydroxytestosterone in both wild-type and TNF-$\alpha^{-/-}$ mice (1.87±0.18 to 0.57±0.07 and 1.53±0.18 to 0.59±0.08 nmol/mg protein/min, respectively). Furthermore, CYP2C11 activity was assessed by its ability to catalyze testosterone to 16α-hydroxytestosterone and 2α-hydroxytestosterone. The formation rate of 16α-hydroxytestosterone and 2α-hydroxytestosterone in wild-type mice was 0.15±0.06 and 0.08±0.05 nmol/mg protein/min, respectively, while that in TNF-$\alpha^{-/-}$ mice was 0.13±0.02 and 0.02±0.01 nmol/mg protein/min, respectively. On the other hand, endotoxin completely suppressed the activity of CYP2C11 in both wild-type and TNF-$\alpha^{-/-}$ mice.

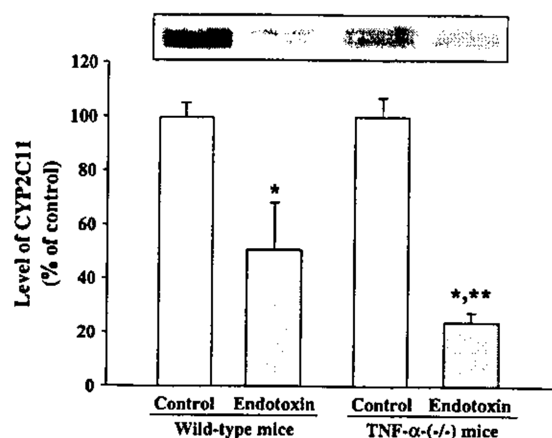

Fig. 4. Effect of endotoxin on hepatic expression of CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

3.5. *Effect of endotoxin on antipyrine elimination in wild-type and TNF-α$^{-/-}$ mice*

Antipyrine is widely used as a tool to evaluate the capacity of drug metabolism in various pathological animal models, since it is almost completely metabolized by the hepatic CYP isozymes in rats (Balani et al., 2002; Kitaichi et al., 1999, 2004). We previously reported that the systemic clearance of antipyrine correlates well with the expression of CYP3A2 and CYP2C11 (Ueyama et al., 2004). Therefore, estimating the plasma concentrations of antipyrine at 3 h after injection in wild-type and TNF-α$^{-/-}$ mice treated 24 h earlier with endotoxin or saline, we found that the plasma concentration of antipyrine in the former was 0.23±0.01 μg/ml, and its value had been increased twofold by the injection of endotoxin (0.45±0.08 μ.g/ml). On the other hand, the concentration in TNF-α$^{-/-}$ mice was 0.40±0.02 μg/ml, and its value had been increased threefold by injection of endotoxin (1.26±0.19 μg/ml). Although no marked difference in the plasma concentration of antipyrine was observed between untreated wild-type mice and TNF-α$^{-/-}$ mice, a significant difference was observed between endotoxin-treated wild-type mice and TNF-α$^{-/-}$ mice.

3.6. *Effect of endotoxin on plasma levels of nitrate/nitrite (NO_x) in wild-type and TNF-α$^{-/-}$ mice*

We previously reported that plasma levels of NO_x in rats 24 h after the injection of endotoxin were significantly higher than those in untreated rats (Kitaichi et al., 1999). We then measured plasma levels of NO_x in wild-type and TNF-α$^{-/-}$ mice 24 h after the injection of endotoxin or saline. As shown in Fig. 5, no significant differences in the constitutive levels of NO_x in plasma were observed between wild-type and TNF-α$^{-/-}$ mice. Endotoxin significantly increased the plasma levels of NO_x by 15-fold and 4-fold in wild-type and TNF-α$^{-/-}$ mice, respectively. The plasma levels of NO_x in endotoxin-treated wild-type mice were 3.5-fold higher than those in endotoxin-treated TNF-α$^{-/-}$ mice.

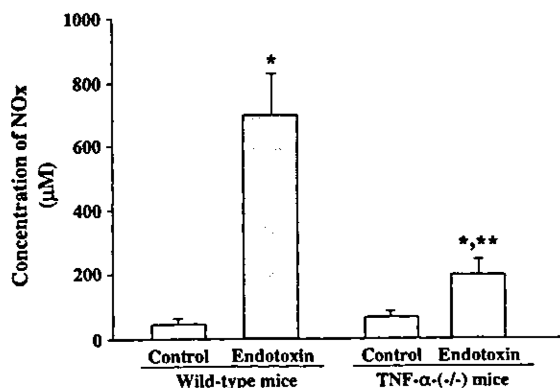

Fig. 5. Effect of endotoxin on plasma concentrations of NO_x in wild-type and TNF-α$^{-/-}$ mice. Values are shown as mean±S.E.M. (n=3). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

4. Discussion

Endotoxin is known to increase the levels of cytokines, including TNF-α, and the elevation of these cytokines might play an important role in endotoxin-induced changes in certain transporter-mediated biliary excretion systems (Hirsch-Ernst et al., 1998; Simpson et al., 1997). We previously reported that the expression of P-glycoprotein mRNA (mdr1a mRNA) in the liver of rats treated 6 h earlier with endotoxin declined, and returned to control levels after 24 h, and that pentoxifylline, which inhibits the over-production of TNF-α, ameliorated the endotoxin-induced reduction in the P-glycoprotein-mediated hepatobiliary excretion of rhodamine 123, which is transported specifically by P-glycoprotein (Ando et al., 2001). These results suggest that TNF-α plays an important role in regulating the expression and function of P-glycoprotein. In the present study, we focused on TNF-α and selected mice with a targeted disruption of the gene encoding TNF-α (Taniguchi et al., 1997).

First, we examined the role of TNF-α in the endotoxin-induced down-regulation of hepatic P-glycoprotein. A significant down-regulation of hepatic P-glycoprotein was observed in wild-type mice at 6 h, but not at 24 h, after the injection of endotoxin. This down-regulation was in good agreement with that seen in our previous studies using rats (Ando et al., 2001), and is further supported by in vitro studies demonstrating that TNF-α reduces the expression of *mdr1* gene in human colon carcinoma cells (Walther and Stein, 1994). In addition, the present results may be supported by our previous study showing that the net biliary excretion of doxorubicin, a substrate of P-glycoprotein, was significantly reduced by the down-regulation of hepatic P-glycoprotein in rats treated with Shiga-like toxin II from *Escherichia coli* O157:H7, which induces an overproduction of TNF-α (Foster et al., 2000; Hidemura et al., 2003). In contrast, it has been reported that endotoxin has no effect on the expression of hepatic P-glycoprotein in TNF-α$^{-/-}$ mice at either 6 or 24 h after injection. We then measured plasma concentrations of doxorubicin to confirm whether the function of P-glycoprotein in TNF-α$^{-/-}$ mice treated 6 h earlier with endotoxin persists; no significant differences in the plasma concentrations of doxorubicin were observed between endotoxin-treated and untreated TNF-α$^{-/-}$ mice, a finding that was in good agreement with the results of Western blot analysis reported previously (Zhao et al., 2002). It is likely that endotoxin-treated TNF-α$^{-/-}$ mice maintain the normal function of P-glycoprotein. Consequently, these results strongly suggest that endogenous TNF-α plays a crucial role in the down-regulation of hepatic P-glycoprotein by endotoxin. However, whether only TNF-α alone is involved in the down-regulation of P-glycoprotein remains to be established.

It has been reported that TNF-α, which is implicated as an important mediator of the pathophysiological effects of