CD14 transgenic mice expressing membrane and soluble forms: comparisons of levels of cytokines and lethalities in response to lipopolysaccharide between transgenic and non-transgenic mice

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Abstract

Two different metallothionein promoter–mouse CD14 fusion genes were constructed. The membrane form of the CD14 fusion gene, designated M14M, contained the full-length CD14 cDNA sequence, whereas the soluble form of the fusion gene, designated M14S, was truncated to lack the sequence for the phosphatidylinositol-anchoring site. Expression of transgenic RNA in M14M and M14S mice on the basal diet was abundant in the liver. After maintenance with water containing ZnSO₄ (50 mM) for 4 days, expression of transgenic RNA in M14M and M14S mice was strong in the small intestine. Immunohistochemical analysis demonstrated CD14 expression in these organs in M14S and M14M mice. Levels of CD14 in sera from M14S mice after zinc administration were significantly higher than these animals maintained with normal water, M14M mice after zinc administration and non-transgenic mice. Sera from M14S and M14M mice after stimulation with lipopolysaccharide LPS (LPS) demonstrated significantly lower levels of tumor necrosis factor-α and IL-6 than those from non-transgenic mice. Lethality in endotoxin shock produced by i.p. injection of 30–40 µg/g body wt LPS was not different between M14S, M14M and non-transgenic mice. However, survival rates in the lethal Shwartzman reaction induced by priming and challenge injections of LPS were significantly higher in M14M and M14S mice than in non-transgenic mice.

Introduction

The phosphatidylinositol-linked cell surface molecule CD14, which is mainly expressed in myelomonocytic cells (1–3), serves as a receptor for lipopolysaccharide (LPS) with the aid of a lipid transfer protein LPS-binding protein, triggering signal transduction pathways that lead to activation of myelomonocytic cells (4,5). The CD14 system is highly sensitive and can detect nanogram amounts of LPS. Such a cellular event recognized by the system is thought to be the central event in initiation of endotoxin shock, in which products of macrophages such as cytokines, oxygen radicals and arachidonic acid metabolites play major roles. Such a proposal could be substantiated by analyzing the function of CD14 in vivo. Artificial control of CD14 expression in mice may provide insight into the roles of CD14 in vivo and in endotoxin shock. However, much lower sensitivity of mice to LPS compared to human could cause difficulty of judgement of lethality in response to LPS. Nevertheless, one example using CD14 transgenic mice expressing human CD14 in monocytic cells has suggested in vivo roles of CD14, although the results are preliminary (6). Furthermore, recent evidence in CD14 disrupted mice have shown that such mice are highly resistant to endotoxin shock and bacterial dissemination (7).

There are two other reasons for examining the in vivo roles of CD14 using transgenic techniques. First, a number of reports concerning cell-surface receptors including receptors for tumor necrosis factor (TNF)-α have shown that soluble
forms of these receptors inhibit binding of ligands by competing with cell-surface receptors (8,9), suggesting that the soluble form of CD14 may also act in the same way. In support of this idea, human recombinant soluble CD14 (sCD14) has been reported to prevent mortality in mice treated with LPS (10). However, it is unknown whether sCD14 simply plays a role in the inhibition of endotoxin shock because sCD14–LPS has been shown to interact with an as yet unidentified receptors on endothelial cells to induce activation of the cells (11–14). Second, CD14-transfected Chinese hamster ovary cells can be stimulated with LPS, inducing NF-κB translocation and production of prostaglandin (15,16). These results indicate that CD14 expressed in non-myelomonocytic cells can trigger cell activation by LPS, although it is unclear whether such activation plays a role in vivo.

In the present study, we produced transgenic mice carrying the native or truncated form of the mouse CD14 gene under the control of the metallothionein promoter which regulates expression of CD14 in organs other than myelomonocytic cells (17). We examined the responses of these mice in response to LPS and discussed the in vivo roles of membrane and sCD14 in vivo.

**Methods**

**Plasmid construction**
To derive transgenic lines expressing the membrane and sCD14 products, we constructed two different metallothionein promoter–CD14 fusion genes. The structures of M14M and M14S are shown in Fig. 1. M14M was constructed using

![Diagram of CD14 transgenic mice and their responses to LPS](image)

**Fig. 1.** Structures of the transgenes. (1) M14M and (2) M14S. The open regions corresponds to the metallothionein promoter sequence. The filled regions represent exons of the CD14 gene. The shaded region corresponds to the SV40 polyadenylation sequence.

pUC19. Standard recombinant techniques were used to introduce (i) a 1.4 kb HindIII–BamHI genomic fragment of mouse CD14 containing the 55 bp of the first exon, the intron and part of the second exon containing the entire coding region and 164 bp of the 3′ untranslated region; (ii) a 1 kb EcoRI–HindIII fragment of mouse metallothionein promoter, derived from pMGH kindly supplied by Dr Evans (18); and (iii) a 300 bp BamHI fragment of SV40 polyadenylation signal sequence. M14S was generated from M14M by introducing one nucleotide substitution of a T for G 117 bp upstream of the stop codon, creating a new stop codon.

**Production and analysis of transgenic mice**
M14M and M14S DNA were prepared for microinjection by digestion with 5 U (each) of EcoRI and BamHI per 10 μg of DNA for 1 h. The DNAs were electrophoresed through a 1% agarose gel. Hybrid inbred (C57BL/6×CBA)F1 mice (Shizuoka Laboratory, Shizuoka, Japan) were used as stud males, embryo donors and mature females for breeding. Outbred ICR mice (Shizuoka) were used as vasectomized males and pseudopregnant females. The procedure for generating transgenic mice has been described previously (19). F1 female mice were mated the night before injection with F1 males and eggs were prepared for injection as described previously (19). Briefly, 0.5–1 pl of DNA solution was injected into the male pronucleus. Following microinjection, viable eggs were washed once in M2 media and transferred to the oviducts of pseudopregnant ICR mice. Transgene-positive animals were identified by PCR amplification or Southern blot analysis of tail tip-derived DNA using the internal 1.5 kb
HindIII–SacI fragment as a probe (20). Since the transgenes carried a single SacI site, the detection of strongly hybridizing species in the 1.4 kb fragment was diagnostic for transgene integration. Three founder mice were produced which carried the M14M sequence (M14M1, M14M2 and M14M9) and four founders were identified with the M14S transgene (M14S1, M14S2, M14S3 and M14S4). The founders were bred with normal C57BL/6 mice and transmitted the transgenes to their progeny in a Mendelian fashion with the exception of M14M1. The M14M1 founder was presumed to be mosaic as only two of 48 offspring were transgenic; in each case subsequent inheritance was as predicted for a normal autosomal locus. Southern blot analysis of tail DNA demonstrated multiple copies of the transgene (between five and 40 copies depending on the founder). M14M2 and M14S1 lines were used as M14M and M14S lines respectively in the present paper.

Northern blot analysis

Northern blot analysis was performed as described previously (2). Briefly, total RNA prepared from organs was electrophoresed through 1.5% agarose/6% (v/v) formaldehyde gels and blotted onto nylon membranes. The membranes were exposed to UV for 7 min, and then prehybridized and hybridized with 3–5 × 10⁶ c.p.m./ml of 32P-labeled CD14 cDNA (2,21).

Immunohistochemistry

Immunostaining was carried out on sections prepared from Carnoy-fixed organs using an indirect peroxinperoxidase staining technique as described previously (22). Briefly, binding of polyclonal rabbit anti-CD14 or mAb rmC5-3 to sections was detected using horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-rat IgG (H and L chain specific) (Cappel, Durham, NC), followed by substrate. Sections incubated in the absence of primary antibody or with an irrelevant primary antibody were included as negative controls.

Measurement of sCD14

Western blot analysis was performed for detection of sCD14. Sera were prepared from transgenic and non-transgenic mice before and after maintenance with water containing ZnSO4 (50 mM) for 4 days and LPS stimulation. The sera were electrophoresed under reducing conditions in SDS on 8–10% polyacrylamide gels. Proteins were electroblotted to nitrocellulose membranes in Tris–glycine, 20% methanol and 0.9% NaCl, pH 8.2, with 1% dry milk and 0.05% Na2SO4, rmC5-3 diluted in blocking buffer was used to stain the blots and bound antibody was detected by using peroxidase-labeled rabbit anti-rat IgG (H and L chain specific) (Cappel) (1:500) for 30 min at 37°C. Blots were developed by an ECL technique (ECL kit, Amersham Japan, Tokyo, Japan) according to the manufacturer’s instructions. Blots were quantified by densitometric analysis of each protein band compared to a background reading from each membrane using the NIH Image analysis system and the data is expressed in relative amounts. Data are presented as mean ± SD of three independent experiments.

Measurement of TNF-α and IL-6

TNF-α and IL-6 were measured using mouse TNF-α and IL-6 ELISA kits (Genzyme, Cambridge, MA).

Induction of endotoxin shock

Age-matched heterozygote transgenic and non-transgenic mice used in these experiments were in the fourth backcross to C57BL/6. Mice were injected i.p. with LPS (30 or 40 µg/g) from Escherichia coli (Sigma, St Louis, MO).

Induction of the generalized Shwartzman reaction

Age-matched heterozygote transgenic and non-transgenic mice used in these experiments were in the fifth backcross to C57BL/6 (BL6). The generalized Shwartzman reaction was elicited by two consecutive injections of Salmonella enteritidis LPS (Sigma) (23). The priming injection (0.25 µg/g) was given in the footpad and was followed 24 h later by a challenge LPS injection (20 or 25 µg/g) given i.v. Each experimental group included 10–15 mice.

Statistical analysis

Values are expressed as mean ± SD. Significant differences were determined by Student’s unpaired-test and P < 0.05 was considered statistically significant. Survival data were compared for statistical significance using χ² analysis.

Results

Expression of the CD14 transgene

The tissue specificity of transcription from the metallothionein promoter was analyzed previously, and liver and kidney were the sites of constitutive expression in the mouse lines. The metallothionein promoter is strongly inducible by metal administration in these tissues, although rate of induction differs among tissues and time course and levels of induction are different between different metals (18). To investigate the expression of the CD14 transgene, total RNA was extracted from a variety of tissues of 4-week-old transgenic animals.
CD14 transgenic mice and their responses to LPS

before and after zinc administration and subjected to Northern blot analysis. The size of predicted transgenic mRNA in M14S lines was 1.8 kb, whereas that of non-transgenic mice was 1.5 kb. Expression of transgenic RNA in M14S mice on the basal diet was abundant in the liver, slight in lung, spleen, kidney and testis, and barely detectable or absent from brain, thymus, heart, muscle, small intestine and colon (data not shown). After maintenance with water containing ZnSO₄ (50 mM) for 4 days, expression of transgenic mRNA in M14S mice was strong in the small intestine, followed by the liver and testis. Low levels of expression were observed in lung and colon (Fig. 2). Expression patterns on the basal diet and following zinc administration in M14M mice were similar to those in M14S mice.

Immunohistochemical analysis of CD14 expression was performed for various organs from M14M and M14S mice. The cytoplasm of the liver from these mice was stained with CD14. Intensity of the staining varied among cells. After maintenance with water containing 50 mM ZnSO₄ for 4 days, the staining intensity increased significantly. The small intestine in M14S mice showed no staining for CD14. After maintenance with water containing ZnSO₄, however, marked staining was observed in the luminal portion of the small intestinal columnar epithelium from these mice (Fig. 3). Similar results were observed in M14M mice.

Levels of sCD14 and cytokine production in transgenic and non-transgenic mice
sCD14 and cytokine production in transgenic and non-transgenic mice M14M and M14S lines used in these experiments were in the fourth backcross to BL6. Relative serum levels of sCD14 were measured by Western blot analysis. Levels of CD14 in sera from M14S mice after maintenance with water containing 50 mM ZnSO₄ for 4 days were high compared with those from non-transgenic and M14M mice after zinc administration (Fig. 4A and B). The level was increased after i.p. injection with LPS (20 µg/mouse). In contrast, the levels of sCD14 in sera from M14M and non-transgenic mice were largely up-regulated, reaching a peak at 12 h in non-transgenic mice and 4 h in M14M mice respectively after LPS stimulation. sCD14 from M14S, M14M and non-transgenic mice were capable of binding LPS (data not shown).
sCD14 is expected to compete with membrane-bound CD14 for LPS binding, resulting in decreased production of TNF-α and IL-6. M14S and non-transgenic mice after maintenance with water containing 50 mM ZnSO₄ received i.v. injection of LPS (20 µg/mouse). Sera prepared from mice 1 and 4 h after LPS injection were then subjected to TNF-α and IL-6 assays. Sera from M14M and M14S mice prepared from 1 h after LPS injection demonstrated significantly lower levels of TNF-α than those from non-transgenic mice (Fig. 5).
CD14 transgenic mice and their responses to LPS

The IL-6 level in M14S mice 1 h after LPS injection showed a significant decrease and that in M14M mice showed a lower tendency, whereas that in both M14S and M14M mice 4 h after LPS injection showed a significant decrease.

**Lethality in transgenic and non-transgenic mice in endotoxin shock and the generalized Schwartzman reaction**

Similar mice used for the measurement of levels of sCD14 and cytokines in sera were used for endotoxin shock experiments. Groups of 15 transgenic and non-transgenic mice received i.p. injections of LPS from *E. coli*. Significant differences in lethality between M14S, M14M and non-transgenic mice injected with LPS (30 or 40 µg/g) were not observed (Table 1).

Transgenic and non-transgenic mice were used after maintenance with water containing 50 mM ZnSO₄. Groups of 10–15 transgenic and non-transgenic mice received two consecutive injections of LPS from *S. enteritidis*. The priming injection was given in the foot pad and was followed 24 h later by the i.v. challenge injection. The reaction in non-transgenic mice was lethal within 24 h only if the mice received both the preparative and the challenge injections (100% mortality). These mice showed pilo-elevation, weakness, diarrhea, bleeding at the conjunctiva, nose tip, mouth, anus and tail end, and paralysis. Most mice died within 24 h. A single footpad or a single i.v. injection was ineffective. In contrast, the priming and challenge injections resulted in less marked clinical symptoms and 80% survival in M14M mice 48 h after challenge injection, whereas the same injections resulted in 40% survival in M14S mice (Fig. 6A and B). The condition giving 87% lethality in wild-type mice abolished lethality in M14M mice, whereas it greatly reduced lethality in M14S mice.

**Table 1. Lethality of mice with a single i.p. injection of LPS**

<table>
<thead>
<tr>
<th>Mice</th>
<th>LPS dose (µg/g)</th>
<th>No. of mice dead/tested</th>
<th>Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL6</td>
<td>30</td>
<td>8/15</td>
<td>53</td>
</tr>
<tr>
<td>BL6</td>
<td>40</td>
<td>14/15</td>
<td>93</td>
</tr>
<tr>
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<td>7/15</td>
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<tr>
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<td>53</td>
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<tr>
<td>M14S</td>
<td>40</td>
<td>13/15</td>
<td>87</td>
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</tbody>
</table>

Each mouse received an i.p. injection of LPS.
CD14 transgenic mice and their responses to LPS

Fig. 6. Lethality of non-transgenic, M14M and M14S mice in the generalized Schwartzman reaction. The Schwartzman reaction was elicited in mice by two consecutive injections of LPS. The priming injection (0.25 μg/g, footpad) was followed 24 h later by a 20 or 25 μg/g i.v. challenge injection. (A) n = 10 per group. (B) n = 15 per group. Open squares/dashed line, non-transgenic mice (25 μg/g LPS); filled circles/dashed line, M14M mice (25 μg/g LPS); open squares/solid line, non-transgenic mice (20 μg/g LPS); filled circles/solid line, M14M mice (20 μg/g LPS); open circles, dashed line, M14S mice (25 μg/g LPS); open circles/solid line, M14S mice (20 μg/g LPS). *P < 0.01; **P < 0.03.

Discussion

CD14 is mainly expressed in myelomonocytic cells. Using transgenic techniques, CD14 was abnormally expressed in cells other than leukocytes. Expression of CD14 was observed in liver irrespective of zinc administration. The expression in liver may be efficiently regulated by zinc because the conventional diet contains basal amounts of zinc. In contrast, the small intestine expressed CD14 only after zinc administration, this expression being very strong. Expression features of CD14 in these cells both in M14M and M14S mice were very similar when examined by immunohistochemistry, suggesting staining of precursor proteins in the cytoplasm in these cells.

The expression in liver parenchymal cells was patchy, whereas that in small intestine was found in the cells in the luminal area, suggesting different regulation of expression of transgenic CD14 between cells or by position. Although the reasons for different features of expression among hepatic cells are unclear, the liver in mice injected with LPS or Propionibacterium acnes shows homogeneous and membranous expression of CD14 (manuscript in preparation). Therefore, all liver cells in transgenic mice are thought to be potentially active for CD14 expression. The expression of CD14 in the small intestine may be the direct effect of zinc in the drinking water on intestinal cells because the expression was found exclusively in luminal cells and the expression in the small intestine in mice following i.p. injection of zinc was low (results not shown).

The truncated transgene used for M14S was designed to produce CD14 lacking the putative membrane-anchoring region. As expected, the level of sCD14 in M14S mice was constitutively high. Furthermore, the level was up-regulated after LPS stimulation. The level of sCD14 in M14M mice tended to be high and up-regulated after LPS stimulation. However, this features of sCD14 production was essentially similar to those in non-transgenic mice, although the time course of production was different. sCD14 in M14S and M14M mice was functional because it was capable of binding LPS (data not shown). Plasma TNF-α levels were significantly lower in M14S and M14M mice than non-transgenic mice after LPS stimulation. Plasma IL-6 levels were significantly lower in M14S than non-transgenic mice after LPS stimulation, whereas a similar tendency was observed in M14M mice. Therefore, the down-regulation of cytokine production in M14S mice could be ascribable to the competitive LPS-binding by sCD14. Low levels of cytokine production was also found in M14M mice, main reasons of which may be due to clearance of LPS by the membrane CD14 in heterotopic cells, although sCD14 plays a role in some extent.

Differences in lethality following a single i.p. injection of large dose of LPS (30–40 μg/g) were not observed between transgenic and non-transgenic mice. Even overexpressed CD14 irrespective of membrane or soluble form in transgenic mice might not be able to handle such large amounts of LPS. In such conditions, CD14-independent signaling pathways should take place (5,24), resulting in no differences in lethality between transgenic and non-transgenic mice.

The generalized Schwartzman reaction, in which a priming dose of LPS is injected intradermally in the foot pad, is followed 24 h by an i.v. challenge injection of LPS (23). The priming injection only requires a small dose of LPS, which is not lethal per se. Using this model, we could observe differences in mortality between transgenic and non-transgenic mice. Lethality in M14S mice was significantly reduced, although 60% of them died by 48 h. On the other hand, M14M mice were unequivocally resistant to the lethal Schwartzman reaction. Several reports indicated that non-myeloid cells such as Chinese hamster ovary cells or pre-B cells transfected with CD14 can transduce LPS signaling via CD14 (15,25). Furthermore, LPS can stimulate endothelial cells or glioblastoma cell lines by sCD14 (29). However, liver and intestinal cells expressing CD14 in M14M mice may not be involved in cytokine production. Rather, CD14 in these cells is likely to clear up circulating LPS, abrogating the effect of a priming injection of LPS to myeloid and Kupffer cells. In addition, intestinal CD14 may play an important role in elimination of endogenous LPS into the circulation because of trapping of intestinal LPS. The effect of sCD14 is still controversial. Recombinant human sCD14 has been reported to protect endotoxin shock in mice (10). However, LPS–sCD14 is capable of inducing endothelial cell activation, and enhancing their adherence properties to leukocytes and their capacity to release procoagulant and proinflammatory factors (26). It has been reported that the serum level of sCD14 parallels the severity of endotoxin shock (27). Mortality in M14S mice may reflect such conflicting effects of sCD14 in the generalized Schwartzman reaction: sCD14 in M14S mice may reduce LPS of the priming injection, whereas LPS–sCD14 may provide a critical effect on endothelial cells, particularly at later times after challenge injection.
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Abbreviations

LPS lipopolysaccharide
sCD14 soluble CD14
TNF tumor necrosis factor

References


