Mouse Toll-like Receptor 4-MD-2 Complex Mediates Lipopolysaccharide-mimetic Signal Transduction by Taxol*

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Taxol, an antitumor agent derived from a plant, mimics the action of lipopolysaccharide (LPS) in mice but not in humans. Although Taxol is structurally unrelated to LPS, Taxol and LPS are presumed to share a receptor or signaling molecule. The LPS-mimetic activity of Taxol is not observed in LPS-hyporesponsive C3H/HeJ mice, which possess a point mutation in Toll-like receptor 4 (TLR4); therefore, TLR4 appears to be involved in both Taxol and LPS signaling. In addition, TLR4 was recently shown to physically associate with MD-2, a molecule that confers LPS responsiveness on TLR4. To determine whether TLR4-MD-2 complex mediates a Taxol-induced signal, we constructed transformants of the mouse pro-B cell line, Ba/F3, expressing mouse TLR4 alone, both mouse TLR4 and mouse MD-2, and both mouse MD-2 and mouse TLR4 lacking the cytoplasmic portion, and then examined whether Taxol induced NFκB activation in these transfectants. Noticeable NFκB activation by Taxol was detected in Ba/F3 expressing mouse TLR4 and mouse MD-2 but not in the other transfectants. Coexpression of human TLR4 and human MD-2 did not confer Taxol responsiveness on Ba/F3 cells, suggesting that the TLR4-MD-2 complex is responsible for the species specificity with respect to Taxol responsiveness. Furthermore, Taxol-induced NFκB activation via TLR4-MD-2 was blocked by an LPS antagonist that blocks LPS-induced NFκB activation via TLR4-MD-2. These results demonstrated that coexpression of mouse TLR4 and mouse MD-2 is required for Taxol responsiveness and that the TLR4-MD-2 complex is the shared molecule in Taxol and LPS signal transduction in mice.

Taxol, a diterpene purified from the bark of the Western yew (Taxus brevifolia) (1), is an antitumor agent that blocks mitosis by binding and stabilizing microtubules (2, 3). Ding et al. (4) found that Taxol induces the secretion of tumor necrosis factor and down-regulation of tumor necrosis factor receptors in murine macrophages. Although the structure of Taxol is quite different from that of lipopolysaccharide (LPS),1 Taxol has been shown to possess many LPS-like activities, such as tyrosine phosphorylation of microtubule-associated protein kinase (5), induction of LPS-inducible gene expression (6), and activation of NFκB (7). Interestingly, Taxol mimics the actions of LPS on murine macrophages but not on human LPS-responsive cells including macrophages (8, 9).

LPS contains polysaccharide and lipid A portion, the latter of which mediates many LPS responses (10). Several synthetic and natural lipid A analogs, which lack LPS-like activities, have been shown to retain the ability to block LPS-induced cellular responses (11–13). Taxol-induced signaling events in murine macrophages are blocked by some of these LPS antagonists, suggesting that LPS and Taxol share a receptor or signaling molecule (14). Although the target of the antagonists was not defined well, membrane-bound CD14 (mCD14), which has been demonstrated to be involved in LPS-induced signaling events on macrophages (15), might not be the target, because the LPS antagonists suppress LPS-induced signaling under conditions in which it does not block LPS binding to mCD14 (16). These findings are consistent with the observation that Taxol signaling was blocked by an LPS antagonist in a mutant macrophage cell line defective in mCD14 expression (17). Identification of molecules shared in LPS- and Taxol-induced signaling will provide a new insight into the development of anti-inflammatory drugs.

The LPS-mimetic activity of Taxol was not observed in macrophages from a spontaneous LPS-hyporesponsive mutant, C3H/HeJ mice (4–7). Analysis of recombinant inbred mice showed that the genes controlling the responses to LPS and Taxol were closely linked (4). The Lps gene, which has been shown to be responsible for LPS hyporesponsiveness in C3H/HeJ mice, was recently mapped to the Toll-like receptor (Tlr) 4. TLR4 from C3H/HeJ mice has a point mutation that causes a failure to activate NFκB (18–20). TLRs constitute a mammalian transmembrane protein family and are similar to Drosophila Toll in that they have extracellular domains containing leucine-rich repeats and a cytoplasmic portion homologous to the intracellular signaling domain of the type 1 interleukin-1 receptor (21, 22). Studies over the past few years have demonstrated that Drosophila Toll and mammalian TLRs play crucial roles in innate immune recognition (23). Although C3H/HeJ and generated TLR4-deficient mice show LPS hyporesponsiveness (20), expression of TLR4 is not sufficient to confer LPS responsiveness on the human embryonic 293 cell line and the mouse pro-B cell line, Ba/F3, implying a lack of a factor in the transformants (24, 25). Expression of MD-2, a molecule that physically associates with TLR4 on the cell surface, with TLR4 has been demonstrated to confer LPS responsiveness on Ba/F3, which expresses neither TLR4 nor MD-2 (25). These recent

1 The abbreviations used are: LPS, lipopolysaccharide; mCD14, membrane-bound CD14; TLR, Toll-like receptor; Me3SO, dimethyl sulfoxide; mTLR4, epitope-tagged mouse TLR4; mMD-2, epitope-tagged mouse MD-2; EMSA, electrophoretic mobility-shift assay; mTLR4, epitope-tagged mTLR4 lacking the cytoplasmic portion; hTLR4, epitope-tagged human TLR4; hMD-2, epitope-tagged human MD-2.
findings prompted us to determine whether the TLR4-MD-2 complex is the shared molecule in Taxol and LPS signaling. In this paper we demonstrate that the mouse TLR4-MD-2 complex mediates LPS-mimetic signal transduction by Taxol.

EXPERIMENTAL PROCEDURES

Reagents—RPMI 1640 medium and Taxol from Taxus brevifolia were purchased from Sigma. Fetal bovine serum was purchased from Atlanta Biologicals. LPS prepared from Escherichia coli 0111:B4 was purchased from List Biological Laboratories. B464 was provided by Eisai Co., Ltd. (Tokyo, Japan). Tetra-His™ antibody was purchased from Qiagen. BCA protein assay reagents were purchased from Pierce. All other chemicals used were of reagent grade or better.

cDNAs and Expression Constructs—The cDNA encoding human MD-2 was described previously (25). The cDNAs encoding mouse TLR4 or mouse MD-2 were cloned in our own laboratory and will be described elsewhere.3 Restriction sites (Xhol and BamHI) were introduced by polymerase chain reaction, and the cDNAs were cloned into an expression vector, pEFBOS (26). The DNA fragment encoding the FLAG epitope followed by the 6xHis epitope was introduced into the pEFBOS vector such that all expressed protein bore the FLAG and 6xHis epitope at the C termini. The mouse TLR4Δ DNA encodes the truncated protein in which the cytoplasmic region encompassing C-terminal 134 amino acids (662–835) was deleted.

Species Specificity of TLR4

Requirement of coexpression of mTLR4 and mMD-2 for noticeable NFκB activation by Taxol was also determined by measuring luciferase activity in Ba/mTLR4/mMD2 cells transfected with an NFκB-dependent luciferase reporter plasmid, p55IgeLUC. Taxol increased the luciferase activity in a dose-dependent manner (Fig. 2B). These results showed that the Ba/mTLR4/mMD2 transfectant acquired Taxol responsiveness.

To determine whether a Taxol-induced signal was mediated by the TLR4 cytoplasmic portion, which is thought to be involved in LPS signaling events (21, 23, 28), we measured NFκB activation by Taxol in a Ba/F3 stable transfectant expressing both mMD-2 and epitope-tagged mouse TLR4 lacking the cytoplasmic portion (mTLR4Δ), named Ba/mTLR4Δ/mMD2, by EMSA. As shown in Fig. 1, NFκB was not activated through Taxol stimulation on Ba/mTLR4Δ/mMD2 cells, implying that the TLR4 cytoplasmic portion is required for Taxol signaling, and that expression of mMD-2 without TLR4 on Ba/F3 is not sufficient for Taxol responsiveness. To eliminate the possibility that the difference in Taxol responsiveness between the transfectants was due to the expression levels of TLR4 and MD-2 proteins, we examined the expression level of the tagged proteins by immunoblot analysis. The expression levels of mouse and human MD-2 were confirmed by immunoblotting with Tetra-His™ antibody. Ba/F3 and Ba/F3 stable transfectants were maintained as described previously (27) except for the use of heat-inactivated (56°C for 30 min) fetal bovine serum.

Luciferase Assay—Unless otherwise indicated, cells were inoculated into a 48-well dish (Corning) or a 1.5-mL tube (Trefill) at 2 × 10⁶ cells/500 μL of cell culture medium. Taxol, LPS, and LPS antagonists were added as described in the figure legends. After stimulation at 37°C for 4 h, cells were harvested and lysed in 50 μL of cell culture lysis reagent (Promega Corp.), and then luciferase activity was measured using 5 μL of lysate and 25 μL of luciferase assay substrate (Promega Corp.). The luminescence was quantified with a luminometer (Berthold Japan, Tokyo, Japan).

RESULTS

Coexpression of Mouse TLR4 and Mouse MD-2 Confers Taxol Responsiveness on Ba/F3—Genetic characterization of C3H/HeJ mice suggested that a point mutation in TLR4 affects Taxol responsiveness in mice (4, 18, 19). It was left undetermined whether TLR4 directly mediates Taxol-induced signal transduction in mice (4, 18, 19). We also examined the effect of Taxol on NFκB activation by Taxol in a Ba/F3 stable transfectant expressing both mMD-2 and epitope-tagged mouse TLR4 lacking the cytoplasmic portion (mTLR4Δ), named Ba/mTLR4Δ/mMD2, by EMSA. As shown in Fig. 1, NFκB was not activated through Taxol stimulation on Ba/mTLR4Δ/mMD2 cells, implying that the TLR4 cytoplasmic portion is required for Taxol signaling, and that expression of mMD-2 without TLR4 on Ba/F3 is not sufficient for Taxol responsiveness. To eliminate the possibility that the difference in Taxol responsiveness between the transfectants was due to the expression levels of TLR4 and MD-2 proteins, we examined the expression level of the tagged proteins by immunoblot analysis. The expression levels of mTLR4Δ or mTLR4 protein in Ba/mTLR4, Ba/mTLR4/mMD2, and Ba/mTLR4Δ/mMD2 were similar, and that of mMD-2 protein was higher in Ba/mTLR4Δ/mMD2 cells than in Ba/mTLR4/mMD2 cells (data not shown).

These results, taken together, demonstrate that coexpression of mTLR4 and mMD-2 enables Ba/F3 cells to activate NFκB noticeably by Taxol stimulation. Such a requirement for coexpression of TLR4 and MD-2 has also been reported in the case of LPS responsiveness on Ba/F3 cells (Ref. 25 and Fig. 1A), suggesting that the mTLR4/mMD-2 complex is the shared molecule in Taxol and LPS signaling.

Species Specificity of TLR4-MD-2 Complex as to Taxol Responsiveness—We also examined the effect of Taxol on NFκB activation in a Ba/F3 stable transfectant expressing epitope-tagged human TLR4 (hTLR4), epitope-tagged human MD-2 (hMD-2), and reporter gene p55IgeLUC, named Ba/hTLR4/

2 S. Akashi and K. Miyake, manuscripts in preparation.

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An LPS Antagonist, B464, Blocks Taxol-induced NFκB Activation via mTLR4/mMD-2—To further confirm that the mTLR4/mMD-2 complex is the shared molecule in Taxol and mMD-2, named Ba/hTLR4/mMD2, and in a Ba/F3 stable transfectant expressing mTLR4 and hMD-2, named Ba/mTLR4/hMD2, by EMSA. As shown in Fig. 2C, Taxol induced NFκB activation in Ba/hTLR4/mMD2 cells but not in Ba/mTLR4/hMD2 cells. These results suggest that mouse MD-2, but not human MD-2, is involved in Taxol signaling.

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shown in Fig. 4, luciferase activity was clearly increased by Taxol even in the absence of serum, and the reporter activity was further increased when cells were stimulated by Taxol in medium supplemented with 10% serum. On the other hand, LPS-induced NFκB activation was absolutely dependent on serum. These results show that serum factor(s) is not required for Taxol-induced signaling mediated by mTLR4-mMD-2.

**DISCUSSION**

In this study, we show that Taxol responsiveness is acquired through the coexpression of mTLR4 and mMD-2 on Ba/F3 cells. Furthermore, the LPS antagonist, B464, blocks both LPS- and Taxol-induced signals mediated by mTLR4-mMD-2 complex. Taken together, these results demonstrate that mTLR4-mMD-2 complex mediates the LPS-mimetic activity of Taxol and that the complex is the shared molecule in Taxol and LPS signaling.

The Taxol-induced signal via mTLR4-mMD-2 complex activates NFκB. It has been demonstrated that expression of a constitutively active human TLR4 construct, the extracellular portion of which was replaced by CD4, induced the activation of NFκB and that the induced signal was mediated by adaptor protein MyD88, which associates with the cytoplasmic portion of TLR4, and subsequently by IRAK and TRAF6 (28). Taxol-induced NFκB activation might be mediated by a similar pathway, as we found in this study that the cytoplasmic portion of mouse TLR4 was required for Taxol signaling. The fact that Taxol-induced NFκB activation shares this pathway with LPS signaling might be responsible for its LPS-mimetic activity.

An important question to be addressed is whether TLR4-MD-2 complex can directly recognize extracellular stimulants such as LPS and Taxol. To determine whether mTLR4-mMD-2 complex is a receptor for Taxol, [1H]Taxol binding to Ba/mTLR4/mMD2 cells was compared with that of parental Ba/F3 cells, and we did not detect any significant Taxol binding to Ba/mTLR4/mMD2 cells.3 Previously, we had also examined whether LPS binds to hTLR4-hMD-2, but we did not detect significant LPS binding to hTLR4-hMD-2 complex.2 Thus, the mechanism of recognition of the stimulant by TLR4-MD-2 remains to be elucidated. If mTLR4-mMD-2 were a receptor for Taxol, the binding of only a minute amount of Taxol to mTLR4-mMD-2, undetectable under our binding assay conditions, might be sufficient to induce a signal. Alternatively, another molecule(s) might serve as an initial receptor for Taxol, and then the Taxol receptor complex might be recognized by mTLR4-mMD-2. Recently, several proteins were identified as Taxol-binding proteins, such as CD18 (30) and HSP-90 (31). One or both of these Taxol-binding proteins might serve as an initial receptor and mediate signals to mTLR4-mMD-2 complex.

Interestingly, Taxol-induced signaling was mediated by mTLR4-mMD-2 complex but not by hTLR4-hMD-2 complex, implying that a Taxol-responsive domain exists in mTLR4-mMD-2 complex. Human TLR4 exhibited 69% amino acid sequence identity with mouse TLR4, and human MD-2 exhibited 66% amino acid sequence identity with mouse MD-2.2 The relatively low homology of TLR4 and/or MD-2 between man and mouse may be responsible for the species specificity of Taxol responsiveness on TLR4-MD-2. Our results shown in Fig. 2C suggest that mouse MD-2, but not human MD-2, is important for mediating Taxol signaling. However, the precise role of TLR4 and/or MD-2 in Taxol signaling remains to be elucidated. Because the structure of Taxol is quite different from that of LPS, the molecular mechanism underlying Taxol recognition might be different from that of LPS recognition. Therefore, identification of the Taxol-responsive domain in mTLR4 (or hTLR4-mMD-2 complex) will provide a new clue for understanding the mechanism underlying ligand recognition by TLR4-MD-2 complex. Such fundamental information concerning signal transduction through TLR4-MD-2 complex will also provide a novel insight for the development of anti-inflammatory drugs.

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