Cutting Edge: Gln^{22} of Mouse MD-2 Is Essential for Species-Specific Lipopolysaccharide Mimetic Action of Taxol

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MD-2 associates with the extracellular domain of Toll-like receptor 4 (TLR4) and greatly enhances LPS signaling via TLR4. Taxol, which mimics the action of LPS on murine macrophages, induces signals via mouse TLR4-MD-2, but not via human TLR4-MD-2. Here we investigated the molecular basis for this species-specific action of Taxol. Expression of mouse MD-2 conferred both LPS and Taxol responsive on human embryonic kidney 293 cells expressing mouse TLR4, whereas expression of human MD-2 conferred LPS responsiveness alone, suggesting that MD-2 is responsible for the species-specificity as to Taxol responsiveness. Furthermore, mouse MD-2 mutants, in which Gln22 was changed to other amino acids, showed dramatically reduced ability to confer Taxol responsiveness, although their ability to confer LPS responsiveness was not affected. These results indicated that Gln22 of mouse MD-2 is essential for Taxol signaling but not for LPS signaling. The Journal of Immunology, 2001, 166: 11–14.

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Toll, which encodes a membrane protein involved in Drosophila immunity against fungi (2); TLRs constitute a transmembrane protein family that has leucine-rich repeats in the extracellular portion and a cytoplasmic portion homologous to the intracellular signaling domain of type I IL-1 receptor (3, 4). Involvement of TLR4 in LPS responses was first suggested by the discovery that TLR4 from spontaneous LPS-hyporesponsive mutant mice, C3H/HeJ, has a point mutation that causes an inability to activate NF-κB (5, 6, 7). Subsequently, using generated TLR4-deficient mice, TLR4 was unequivocally demonstrated to be involved in LPS responses (7). Furthermore, TLR4 was suggested to be involved in ligand-specific recognition of LPS (8, 9), although direct binding of TLR4 with LPS on the cell surface has not been demonstrated. However, expression of TLR4 alone is not sufficient for LPS responsiveness to be conferred on a mouse pro B cell line, Ba/F3 cells (10). In addition to TLR4, the expression of MD-2, which associates with the extracellular domain of TLR4, is required for LPS responsiveness to be conferred on Ba/F3 cells (10). In fact, there is a TLR4-MD-2 complex on the surface of mouse peritoneal macrophages, and the complex on the macrophages has been shown to be involved in LPS responses (11).

Taxol, a diterpene purified from the bark of the Western yew (Taxus brevifolia) (12), is an antitumor agent that blocks mitosis by binding and stabilizing microtubules (13, 14). Although the structure of Taxol is quite different from that of the LPS lipid A moiety, which is responsible for many LPS responses (15), Taxol has been shown to exhibit many LPS-mimetic activities as to murine macrophages, such as induction of TNF secretion (16), NO production (17), and NF-κB activation (18). Taxol was suggested to share a receptor and/or signaling molecule with LPS because these LPS-mimetic activities of Taxol were not detected in macrophages from LPS-hyporesponsive C3H/HeJ mice (16–18). Interestingly, the LPS-mimetic activities of Taxol were species specific; Taxol did not mimic the action of LPS on human LPS-responsive cells including macrophages (19, 20). By using stable transfectants expressing MD-2 and/or TLR4, we found that mouse TLR4-MD-2 mediates LPS-mimetic signal transduction by Taxol and that the species-specific LPS-mimetic action of Taxol was based on the species difference between mouse and human TLR4-MD-2 (21). In this study, we investigated the molecular basis for the species-specific LPS-mimetic action of Taxol and found that Gln22 of mouse MD-2 is essential for LPS-mimetic Taxol signaling but not for LPS signaling.

**Materials and Methods**

**Reagents**

Taxol from *Taxus brevifolia* was purchased from Sigma (St. Louis, MO). LPS prepared from *Escherichia coli* 0111:B4 was purchased from List

**Abbreviations used in this paper:** TLR, Toll-like receptor; HEK, human embryonic kidney; cMD-2, chimeric MD-2.

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FBS, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated FBS. The cells were transfected with plasmids (0.3 μg/well) using FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN). Three days after transfection, the cells were cultivated in culture medium containing 0.5% DMSO (medium) or in culture medium containing 0.5% DMSO and 100 ng/ml LPS or 10 μM Taxol for 6 h, and then luciferase activity was measured. Luciferase activity was normalized as to the activity in the cells cultivated in the culture medium containing 0.5% DMSO, and are presented as fold induction. The columns indicate the averages for duplicate wells.

Stable transfectants and cell culture

Human embryonic kidney (HEK) 293 cells stably expressing a recombinant mouse TLR4 bearing a FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and a 6× His epitope at its C terminus were generated by introducing the mouse TLR4 expression construct prepared as described previously (11). Expression of the recombinant mouse TLR4 was confirmed by immunoblotting with Tetra-His Ab (Qiagen, Chatsworth, CA). The stable HEK293 transfectant expressing the recombinant mouse TLR4 and HEK293 cells were transfected with p55Igk luc, an NF-κB-dependent luciferase reporter construct (22), the resultant stable transfectants being named 293/luc and 293/mTLR4/luc, respectively. Introduction of p55Igk luc was confirmed by sequencing with an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA). The stuffer region of pEFBOS was eliminated by digestion with XhoI and NotI, and the resulting construct was named pEFBOS(−).

Results and Discussion

Stable HEK293 transfectant expressing mouse TLR4 acquires Taxol responsiveness through the expression of mouse MD-2, but not through that of human MD-2

Expression constructs

Human (10) or mouse (11) MD-2 cDNA was cloned into the XhoI and NotI sites of an expression vector, pEFBOS (23), by PCR-based introduction of restriction sites. Human/mouse chimeric MD-2 (cMD-2) cDNAs and mutant MD-2 cDNAs were generated by PCR-based overlap extension (24). The sequences of the PCR primers are available upon request. cMD-2 cDNAs and mutant MD-2 cDNAs were cloned into the XhoI and NotI sites of plasmid DNA Purification System (Promega, Madison, WI).

Transient transfection

Cells (3 × 10⁶) were seeded into the wells of a 24-well dish (Costar, Cambridge, MA), containing 1 ml of cell culture medium. After cultivation overnight, the cells were transfected with plasmids (0.3 μg/well) using FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN). The plasmids used for transfection were purified with a Wizard PureFec Plasmid DNA Purification System (Promega, Madison, WI).

Luciferase activity assay

Luciferase activity in cell lysates was measured with a luciferase assay system (Promega) as described previously (21).

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expression of human or mouse MD-2 confers LPS or Taxol responsiveness on 293/mTLR4/luc cells by measuring NF-κB activation. As shown in Fig. 1, 293/mTLR4/luc cells expressing mouse MD-2 responded well to both Taxol and LPS stimulation, but 293/mTLR4/luc cells expressing human MD-2 responded to LPS stimulation but not to Taxol stimulation. These results show that human MD-2 does not have the ability to confer Taxol responsiveness on mouse TLR4, although it has the ability to confer LPS responsiveness on mouse TLR4.

Mouse/human cMD-2 shows reduced ability to confer Taxol responsiveness

The amino acid sequence (160 aa) encoded by mouse MD-2 cDNA exhibited 64% identity with that (160 aa) encoded by human MD-2 cDNA (11). To determine whether there is a domain(s) of mouse MD-2 responsible for conferring Taxol responsiveness on mouse TLR4, constructs for expressing mouse/human cMD-2 proteins (Fig. 2A) were generated, and their ability to confer Taxol responsiveness was examined. The 293/mTLR4/luc cells expressing cMD2-2, -3, -4, -5, or -6 did not respond to Taxol stimulation, and those expressing cMD2-1 slightly responded to Taxol stimulation (Fig. 2B). In contrast, all of the transfectants expressing cMD-2 proteins responded to LPS stimulation (Fig. 2B). These results suggested that the ability of mouse MD-2 to confer Taxol responsiveness on mouse TLR4 is not due to a particular domain of mouse MD-2.

Gln22 of mouse MD-2 is an essential residue for Taxol signaling

Although only nine amino acid residues were different between the amino acid sequences of mouse MD-2 and cMD2–1 (Figs. 2A and 3A), the ability of mouse MD-2 to confer Taxol responsiveness was apparently different from that of cMD2–1, the fold induction of reporter activity on Taxol stimulation being 14- and 3.2-fold in 293/mTLR4/luc cells expressing mouse MD-2 and cMD2–1, respectively (Fig. 2B). To determine which of the nine different amino acid residues were important for Taxol signaling, we generated mutant mouse MD-2 expression constructs in which Pro13, Ser18, Glu19, or Gln22 was replaced with the corresponding amino acid sequence (160 aa) encoded by mouse MD-2 in which Pro13, Ser18, Glu19, or Gln22 was changed to Gln. Three days after transfection, the cells were cultivated in culture medium containing 0.5% DMSO and the indicated amount of Taxol (a) or LPS (b) for 6 h, and then luciferase activity was measured. The results shown are the averages for duplicate wells.
acid residue of human MD-2, and then their ability to confer Taxol and LPS responsiveness was examined (Fig. 3B). Although P13S-, S18A-, and E19Q-mouse MD-2 exhibited similar abilities to confer Taxol responsiveness on 293/mTLR4/luc cells to that of mouse MD-2, Q22Y-mouse MD-2 had an apparently lower ability to confer Taxol responsiveness than mouse MD-2, the fold induction of reporter activity on 10 μM Taxol stimulation being 9.4- and 2.7-fold in 293/mTLR4/luc cells expressing MD-2 and Q22Y-mouse MD-2, respectively (Fig. 3B). With regard to the effect of the Taxol concentration on NF-κB activation, 1 μM Taxol induced NF-κB activation in the cells expressing mouse MD-2, whereas 10 μM Taxol was required to induce NF-κB activation in the cells expressing Q22Y-mouse MD-2, just like in the cells expressing cMD2–1 (Fig. 3C). Furthermore, the cells expressing Y22Q-cMD2–1, in which Tyr22 of cMD2–1 was replaced with mouse type Gln22, showed similar sensitivity to Taxol to that of the cells expressing mouse MD-2 (Fig. 3C). In contrast, the sensitivity to LPS was similar between the cells expressing mouse MD-2 and those expressing Q22Y-mouse MD-2 (Fig. 3, B and C). These results, taken together, indicate that Gln22 of mouse MD-2 is involved in the species-specific LPS-mimetic action of Taxol.

To determine whether Gln22 is essential for Taxol signaling or Tyr22 specifically inhibits Taxol signaling, several mutants of mouse MD-2 were generated by replacing Gln22 with other amino acids and then their ability to confer Taxol and LPS responsiveness was examined. The 293/mTLR4 cells expressing Q22K-, Q22S- and Q22F-mouse MD-2 did not respond to Taxol (Fig. 4). In contrast, the level of Taxol-induced NF-κB activation in the 293/mTLR4 cells expressing Q22E-mouse MD-2 was similar to that in those expressing mouse MD-2 (Fig. 4). The structural similarity between glutamic acid and glutamine may explain why Q22E-mouse MD-2 has the ability to confer Taxol responsiveness. It is noteworthy that the level of LPS-induced NF-κB activation was similar among these cells expressing mutant mouse MD-2 proteins. These results, taken together, suggest that Gln22 is an essential residue for Taxol signaling.

In this study, we showed that human MD-2 did not have the ability to confer Taxol responsiveness on mouse TLR4 and that the ability of mouse MD-2 was not due to a particular domain of mouse MD-2. Furthermore, we found that Gln22 of MD-2 is essential for Taxol signaling but not for LPS signaling.

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