Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition

Sachiko Akashi, Yoshinori Nagai, Hirotaka Ogata, Masato Oikawa, Koichi Fukase, Shoichi Kusumoto, Kiyoshi Kawasaki, Masahiro Nishijima, Shinichiro Hayashi, Masao Kimoto and Kensuke Miyake

Departments of Immunology and 1Internal Medicine, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501, Japan
2Department of Chemistry, Graduate School of Science, Osaka University, Osaka 560-0043, Japan
3Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Keywords: innate immunity, MD-2, TLR4

Abstract

Toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS). MD-2 is associated with TLR4 and imparts LPS responsiveness to it. Little is known, however, as to whether MD-2 directly regulates LPS recognition by TLR4. To address the issue, we took advantage of a species-specific pharmacology of lipid IVa, an analogue of lipid A. Lipid IVa acted agonistically on mouse (m) TLR4/MD-2 but not on human (h) TLR4/MD-2. Lipid IVa antagonized the agonistic effect of lipid A on hTLR4/MD-2. We examined the chimeric complex consisting of mTLR4 and hMD-2 to ask whether species specificity is conferred by TLR4 or MD-2. hMD-2 was clearly distinct from mMD-2 in the way of influencing LPS recognition by mTLR4. hMD-2 conferred on mTLR4 responsiveness to lipid A but not to lipid IVa. Moreover, lipid IVa acted as a lipid A antagonist on mTLR4 that is associated with hMD-2. Collectively, MD-2 directly influences the fine specificity of TLR4.

Introduction

The innate immune response is the first line of defense against microbial pathogens (1,2). The principal challenge for the immune system is to recognize pathogens and mount an immediate defense response. A wide variety of bacterial components are capable of stimulating innate immunity. These include lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, lipoarabinomannan, lipopeptides and bacterial DNA. LPS is a principal component of Gram-negative bacteria that activates the innate immune system and one of the best-studied molecules (3). Toll-like receptor 4 (TLR4) has been implicated in innate recognition and signaling of LPS (4,5). Mutations of the TLR4 gene lead to hyporesponsiveness to LPS in mice and humans (6–9). Exogenous expression of TLR4, however, does not confer LPS responsiveness on cell lines, suggesting a requirement for an additional molecule (10). We recently cloned MD-2, a molecule that is associated with the extracellular domain of TLR4. Co-expression of MD-2 imparts LPS responsiveness to TLR4 (10,11). Little is known, however, about how MD-2 regulates LPS responsiveness of TLR4.

Humans are distinct from mice in recognition of a lipid A analogue, lipid IVa (12–14). Human cells discriminate the difference between these molecules and respond only to lipid A. Mouse cells do not discriminate between them, and respond to both lipid A and lipid IVa. Recent studies showed that species-specific recognition of lipid IVa can be attributed to the species origin of TLR4 (15,16). The previous two studies claimed from these results that TLR4 directly and physically binds to LPS (15,16). It is, however, not clarified whether MD-2 contributes to the discrimination. This issue allows us to ask whether MD-2 directly regulates LPS recognition by...
TLR4. Here, we established transfectants expressing the chimeric complex consisting of mouse (m) TLR4 and human (h) MD-2, and asked whether this chimeric complex acts as human or mouse.

Methods

cDNAs and expression constructs

The cDNA encoding hMD-2 was described previously (10). All cDNAs were cloned into an expression vector, pEFBOS (17). The DNA fragment encoding the flag epitope followed by the His-tag epitope had been introduced into the pEFBOS vector such that all expressed proteins bear the flag epitope at the C-termini.

Stable transfectants

The plasmids were transfected into Ba/F3 cells by electroporation (18). An NF-κB reporter construct, p55IgκLuc (19), was also introduced, as described previously (10). Expression of TLR4 or MD-2 was confirmed by flow cytometry staining or immunoprecipitation and probing with the anti-flag mAb (Figs 2 and 3, and data not shown).

Reagents

We purchased from Sigma (St Louis, MO) a mAb against the flag epitope M2 and lipid A or LPS derived from Salmonella minnesota (Re595). The tetraacyl lipid A precursor known as lipid IVa (compound 406) was synthesized as described previously (20). Ba/F3 cells were fed in 10% FCS RPMI 1640 supplemented with IL-3 and 50 μM 2-mercaptoethanol.

Luciferase assay

Stable transfectants derived from Ba/F3 were inoculated on to 96-well plates at 1/100,000/well. After 4 h stimulation, cells were harvested, washed and lysed in 100 μl lysis buffer, and luciferase activity was measured using 10 μl lysate and 50 μl luciferase substrate (Toyo Inki, Tokyo, Japan) with a luminometer (Berthold Japan, Tokyo, Japan).

Results

hMD-2 is capable of associating with mTLR4 and enhancing its responsiveness to LPS

Since MD-2 is important for responses to lipid IVa, we sought a role for MD-2 in species-specific recognition of lipid IVa. To address the issue, we established Ba/F3 cells expressing mTLR4/hMD-2 by transfecting hMD-2 into mTLR4-expressing Ba/F3 cells. Cell-surface expression of hMD-2 was detected with a mAb to the flag epitope that had been attached on the hMD-2 molecule (Fig. 2e). mTLR4 was, however, barely detected by the MTS510 mAb that recognizes mTLR4 associated with mMD-2 but not mTLR4 alone (Fig. 2f). We previously showed that, without membrane anchoring via hTLR4, hMD-2 was not detected on Ba/F3 cells (10). It is likely that hMD-2 is anchored via cell-surface mTLR4 that may not be detected by the MTS510 mAb. The association of mTLR4 and hMD-2 was functionally confirmed by responsiveness to lipid A, which was enhanced by hMD-2 co-expression, even more than by mMD-2 (Fig. 4, left panel). Moreover, Kawasaki et al. (22) showed with a transient expression system using HEK293 (21). Although Northern blotting did not reveal endogenous MD-2 expression in Ba/F3 cells (10), we recently found that MD-2 mRNA was detected with RT-PCR (data not shown). A trace of endogenous mMD-2 protein probably explained responses to lipid A or lipid IVa in Ba/F3 cells expressing mTLR4 alone. Kawasaki et al. showed that mTLR4 alone expressed in human kidney cells (HEK293) did not signal LPS at all (22). We used the same line and confirmed that the signaling of lipid IVa as well as of lipid A in the cell line was completely dependent on transiently expressed mMD-2 (data not shown).

Fig. 1. mTLR4/mMD-2 but not hTLR4/hMD-2 responds to lipid IVa. Ba/F3 transfectants expressing a variety of combinations of TLR4 and MD-2 were stimulated with graded doses of lipid IVa for 4 h. Luciferase activities in cell lysates were measured and shown as relative luciferase activity. Similar results were obtained from three independent experiments.

To understand a role of MD-2 in LPS recognition, we studied species-specific responsiveness of the TLR4/MD-2 complex to lipid IVa. Ba/F3 cells expressing mTLR4/MD-2 responded to lipid IVa, whereas those expressing hTLR4/MD-2 did not (Fig. 1). These results are completely consistent with the previous findings that mouse cells but not human cells responded to lipid IVa (12–16). To confirm that the response to lipid IVa was dependent on MD-2, we compared Ba/F3 cells expressing mTLR4 alone or mTLR4/MD-2 (Fig. 1). Although Ba/F3 cells expressing mTLR4 alone showed a low but significant response to lipid IVa, much higher responses were observed in Ba/F3 cells expressing mTLR4/MD-2 (Fig. 1). Responses to lipid IVa were largely dependent on exogenous MD-2.

Ba/F3 cells expressing mTLR4 alone showed low but significant responses to lipid IVa as well as to lipid A (see Fig. 4)
MD-2 and species-specific LPS recognition by TLR4

Fig. 2. Expression of mTLR4 and MD-2 on Ba/F3 transfectants. Mouse TLR4-expressing Ba/F3 cells were further transfected with vectors encoding human or mouse MD-2 as indicated. Human and mouse MD-2 had been tagged with the flag epitope. Expression of MD-2 and the TLR4/MD-2 complex was detected by cell-surface staining with the anti-flag mAb or anti-mouse TLR4 mAb MTS510 followed by goat anti-mouse or rat IgG–FITC respectively. Gray lines depict histograms stained with the second reagent alone.

Fig. 3. Expression of hTLR4 and MD-2 on Ba/F3 transfectants. Human TLR4-expressing Ba/F3 cells were further transfected with vectors encoding human or mouse MD-2 as indicated. Human and mouse MD-2 had been tagged with the flag epitope. Expression of MD-2 and the TLR4/MD-2 complex was detected by cell-surface staining with the anti-flag mAb or anti-human TLR4 mAb HTA125 followed by goat anti-mouse IgG–FITC. Gray lines depict histograms stained with the second reagent alone.

cells that the hMD-2 conferred LPS responsiveness on mTLR4, which alone did not signal LPS in HEK293 cells. We assumed, from these results, that conformation of mTLR4 may be different between mTLR4/mMD-2 and mTLR4/hMD-2, and that the conformation-specific MTS510 mAb did not recognize the mTLR4 conformation with hMD-2 association.

We also established Ba/F3 cells expressing hTLR4/mMD-2. Cell-surface expression of the two molecules was confirmed by the mAb to hTLR4 and to the flag epitope on mMD-2 (Fig. 3e and f). Physical association of hTLR4 and mMD-2 was revealed by co-precipitation of mMD-2 with hTLR4 by a mAb to hTLR4 HTA1216 (data not shown). Despite the physical association, the chimeric hTLR4/mMD-2 complex hardly responded to lipid A (Fig. 4, right panel). Similar results were obtained from a different system, in which transient transfection of hTLR4 and mMD-2 into HEK293 cells did not confer responsiveness to lipid A (data not shown). Due to poor responsiveness of Ba/F3 cells expressing hTLR4 and mMD-2, further studies were focused on those expressing mTLR4/ hMD-2.

We then asked whether the mTLR4/hMD-2 complex behaved as human or mouse by studying responsiveness of mTLR4/hMD-2 to lipid IVa. hMD-2 did not confer on mTLR4 responsiveness to lipid IVa (Fig. 1). It has to be stressed that the lipid IVa response of mTLR4/hMD-2 was lower than mTLR4 alone. Moreover, similar results were obtained from a different system using HEK293 cells expressing mTLR4 alone.

Transient expression of hMD-2 conferred on the HEK293 cells responsiveness to lipid A but not to lipid IVa (data not shown). We next tested antagonistic activity of lipid IVa by adding both LPS from S. minnesota Re595 and lipid IVa. The LPS signal via hTLR4/hMD-2 was completely inhibited by 100 times higher concentration of lipid IVa, whereas no inhibition by lipid IVa was observed with mTLR4/mMD-2 (Fig. 5d and a), consistent with previous studies (12–16). We then asked whether lipid IVa acted as a lipid A antagonist on mTLR4 associated with hMD-2 (Fig. 5b). We obtained similar results with lipid A instead of LPS from S. minnesota Re595 (data not shown). To exclude the possibility that the antagonistic activity of lipid IVa is attributed not to mTLR4/hMD-2 but to mTLR4 alone if expressed on Ba/F3 cells expressing mTLR4 and hMD-2, we examined Ba/F3 cells expressing mTLR4 alone. Lipid IVa showed agonistic activity as shown in Fig. 1 but did not antagonize lipid A (data not shown). hMD-2 association thus changed mTLR4 so that lipid IVa turned antagonistic to lipid A. The other complex consisting of the opposite combination, hTLR4/mMD-2, did not show any significant responses to LPS or lipid IVa (Fig. 5c).

Discussion

We studied how MD-2 influences the LPS responses of TLR4. To address the issue, we took advantage of the fact that mice...
and humans are distinct in terms of discrimination between lipid A and lipid IVa (12–14). Mouse cells but not human cells respond to lipid IVa (12–14). Recent studies attributed the species-specific lipid IVa responses to TLR4 (15, 16). In keeping with this, our experimental system using Ba/F3 transfectants showed that mouse TLR4/MD-2 but not human TLR4/MD-2, responded to lipid IVa (Fig. 1). This system allows us to address a role of MD-2 in species-specific LPS recognition by TLR4. The chimeric complex consisting of mTLR4/hMD-2 responded to lipid A but not to lipid IVa (Fig. 1 and 5). Not only Lipid IVa but also Taxol, an anti-cancer agent, shows species-specific pharmacology between humans and mice (23, 24). Taxol acts agonistically on mouse cells but not on human cells. We previously showed that mouse TLR4/MD-2, but not mTLR4 alone, recognizes and signals Taxol (25). Further, mTLR4/mMD-2 responded to Taxol, whereas mTLR4/hMD-2 did not (22, 25). These results are consistent with the present study and gave another example demonstrating that hMD-2 changed the specificity of mTLR4. The present study provides further evidence for this conclusion. Lipid IVa turned antagonistic to lipid A when acting on mTLR4/hMD-2 (Fig. 5). Collectively, MD-2 regulates the fine specificity of TLR4.

Lipid IVa acts as a lipid A antagonist on mTLR4/hMD-2. Without physical contact with mTLR4/hMD-2, lipid IVa would not be able to antagonize lipid A. The antagonistic activity of lipid IVa therefore indicates that lipid IVa has a physical contact with mTLR4/hMD-2 as lipid A has. Lipid IVa is, however, different from lipid A in subsequent signal transduction. Whereas lipid A triggers an activation signal via mTLR4/hMD-2, lipid IVa does not. hMD-2 association is likely to change the physical contact with mTLR4. Considering that MD-2 regulates the TLR4 conformation (21), MD-2 might, in the presence of LPS, change the TLR4 conformation, which could lead to dimer or multimer formation and thereby to triggering of an activation signal.

Unfortunately, the other chimeric complex consisting of hTLR4 and mMD-2 showed poor responses to lipid A. The way mMD-2 is associated with hTLR4 is likely to be inappropriate for LPS recognition/signaling. Poltrak et al. introduced hTLR4 into a mouse macrophage line derived from C3H/HeJ mice that harbored the TLR4 mutation (15). The C3H/HeJ-derived macrophage line expressing hTLR4 responded to lipid A but not to lipid IVa, showing that TLR4 determines species-specific lipid IVa recognition. We currently do not know the exact reason for the discrepancy between the present study and their study. If hTLR4 was associated with mMD-2 in the C3H/HeJ-derived macrophage line, lipid A responses should be poor, as shown in the present study. The difference may be explained by RP105/MD-1, which is structurally similar to TLR4/MD-2. RP105/MD-1 is expressed on B cells and macrophages but not on 293 cells or Ba/F3 cells. We previously showed that hRP105/MD-1 is able to confer LPS responsiveness on Ba/F3 cells expressing hTLR4 alone (26). Mouse RP105/MD-1, which would be present on the C3H/HeJ-derived macrophage line, might have contributed to the hTLR4-dependent lipid A responses in the study by Pitrak et al. (15). The present study does not necessarily
exclude a role for other molecules such as RP105/MD-1 in LPS recognition by TLR4, but clearly demonstrated that MD-2 is able to directly regulate the fine recognition of LPS by TLR4.

Acknowledgements
This study was supported by Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (Monbukagakusho), Uehara Memorial Foundation, Mitsubishi-Tokyo Pharmaceutical, Inc., and Sankyo Co.

Abbreviations

h human
m mouse
LPS lipopolysaccharide
TLR Toll-like receptor

References
18 Palacios, R. and Steinmetz, M. 1985. IL-3-dependent mouse clones that express B220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell 41:727.