Meso-Diaminopimelic Acid and Meso-Lanthionine, Amino Acids Specific to Bacterial Peptidoglycans, Activate Human Epithelial Cells through NOD1

Akiko Uehara, Yukari Fujimoto, Akiko Kawasaki, Shoichi Kusumoto, Koichi Fukase and Haruhiko Takada

*J Immunol* 2006; 177:1796-1804; doi: 10.4049/jimmunol.177.3.1796
http://www.jimmunol.org/content/177/3/1796

References
This article cites 25 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/177/3/1796.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
**Meso-Diaminopimelic Acid and Meso-Lanthionine, Amino Acids Specific to Bacterial Peptidoglycans, Activate Human Epithelial Cells through NOD1**

Akiko Uehara,* Yukari Fujimoto,† Akiko Kawasaki,† Shoichi Kusumoto,† Koichi Fukase,† and Haruhiko Takada2*

Peptidoglycans (PGNs) are ubiquitous constituents of bacterial cell walls and exhibit various immunobiological activities. Two types of minimum essential PGN structures for immunobiological activities were chemically synthesized and designated as muramylidipeptide; N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), which are common constituents of both Gram-positive and Gram-negative bacteria, as well as most Gram-negative and some Gram-positive bacteria, respectively. Recently, intracellular receptors for MDP and iE-DAP have been demonstrated to be nucleotide-binding oligomerization domain (NOD)1 and NOD2, respectively. In this study, we demonstrated that chemically synthesized positive bacteria, respectively. Recently, intracellular receptors for MDP and iE-DAP have been demonstrated to be nucleotide-binding oligomerization domain (NOD)1 and NOD2, respectively. In this study, we demonstrated that chemically synthesized meso-DAP itself activated human epithelial cells from various tissues, through NOD1 to generate antibacterial factors, PGN recognition proteins and β-defensin 2, and cytokines in specified cases, although the activities of meso-DAP were generally weaker than those of known NOD agonists. However, stereoisomers of meso-DAP, L-L-DAP, and D-D-DAP were only slightly activated or remained inactive, respectively. Synthetic meso-lanthionine, which is another diamino-type amino acid specific to PGN of the specified Gram-negative bacteria, was also recognized by NOD1. In human monocytic cells, in the presence of cytochalasin D meso-DAP induced slightly but significantly increased production of cytokines, although the cells did not respond to meso-DAP in the absence of cytochalasin D. Our findings suggest that NOD1 is a special sentinel molecule, especially in the epithelial barrier, allowing the intracellular detection of bacteria through recognizing meso-DAP or comparable moieties of PGN from specified bacteria in cooperation with NOD2, thereby playing a key role in innate immunity. *The Journal of Immunology, 2006, 177: 1796–1804.

---

*Department of Microbiology and Immunology, Tohoku University Graduate School of Dentistry, Sendai, Japan; and †Department of Chemistry, Graduate School of Science, Tohoku University, Sendai, Japan.

Received for publication December 6, 2005. Accepted for publication April 26, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by Grant-in-Aid 16390519 from the Japan Society for the Promotion of Science. A.U. was supported by Research Fellowship P060931 from the Japan Society for the Promotion of Science.

2 Address correspondence and reprint requests to Dr. Haruhiko Takada, Department of Microbiology and Immunology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. E-mail address: dent-ht0@mail.tains.tohoku.ac.jp

3 Abbreviations used in this paper: PGN, peptidoglycan; MDP, muramylidipeptide; DMP, desmuramylpeptide; DAP, diaminopimelic acid; iE-DAP, γ-D-glutamyl-meso-DAP; NOD, nucleotide-binding oligomerization domain; PGRP, PGN recognition protein; siRNA, short interfering RNA.
prevent possible tissue destruction through by excessive innate immune responses.

In the course of the study to examine the innate immune system in oral epithelium, we found unexpectedly that a commercial DAP specimen activated human oral epithelial cells. Therefore, to further characterize the bacterial moiety recognized by NOD1, we have conducted an exhaustive study using three chemically synthesized stereoisomers of DAP, meso-DAP, LL-DAP, and DD-DAP, in human epithelial and monocytic cells in culture. Furthermore, we examined whether synthetic meso-lanthionine, which is another diamino-type amino acid specific to PGN of some Gram-negative bacteria such as Fusobacterium nucleatum (13), also activated human epithelial cells.

Materials and Methods

Reagents

The synthetic MurNAc-l-Ala-n-isoGln (MDP) was purchased from Protein Research Foundation, Peptide Institute. A synthetic Escherichia coli-type lipid A (LA-15-PP) was purchased from Daiichi Chemical. γ-D-glutamyl-meso-DAP (iE-DAP) was synthesized as previously described (8). Meso-lanthionine was synthesized by coupling of N-benzzyloxy carbonyl-o-cysteine with β-1-chloralalanine according to the procedure reported by Photaki et al. (14). The synthetic DMP, a PGN fragment containing DAP, FK156 (l-lactoyl-l-Ala-γ-D-Glu-meso-DAP-Gly), and FK565 (heptanoly-γ-D-Glu-meso-DAP-n-Ala) (5), was supplied by Astellas Pharmaceutical (formerly Fujisawa and Yamanouchi). Cytochalasin D was purchased from Sigma-Aldrich.

FIGURE 1. Chemically synthesized bacterial PGN fragments. Chemical structure of Lys-type (A) and DAP-type (B) PGN and the respective minimum active moiety reported to sense NOD2 and NOD1 and chemical structures of synthetic DAP isomers and reference synthetic NOD1 agonists (C). NOD2 recognizes MDP (MurNAc-l-Ala-n-isoGln) moiety from Lys-type PGN (A) and probably also MurNAc-l-Ala-n-Glu moiety from DAP-type PGN (B). NOD1 recognizes the DMP moiety containing meso-DAP (B), whose minimum essential structure was reported to be iE-DAP (γ-D-Glu-meso-DAP) until this study. iE-DAP, FK156 (l-lactoyl-l-Ala-γ-D-Glu-meso-DAP-Gly), and FK565 (heptanoly-γ-D-Glu-meso-DAP-n-Ala) were used as reference NOD1 agonists.
Preparation of the isomers of DAP

For separation of the three isomers of DAP (2,6-diaminopimelic acid), α,α-dibenzyl 2-N-(benzylxycarbonyl) 6-N'-t-butoxycarbonyl diaminopimelate, a racemic DAP derivative, was synthesized from DL-DAP (purchased from Sigma-Aldrich). The four isomers (L,L-(2S,6S), D,D-(2R,6R), L,D-(2S,6R), and D,L-(2R,6S)) of the derivative were separated with chiral HPLC column: 1 cm x 25 cm (CHIRALPAK AD-H; Daicel Chemical Industries); mobile phase: n-Hexane/2-propanol 7/3; flow rate: 4 ml/min; detection: UV 254 nm. The retention times and the optical rotation of the compounds were: D,D-(2R,6R): 19.1 min, [α]D = +5.45 (c 0.5, CHCl3); (2R,6S): 34.4 min, [α]D = +0.78 (c 1.0, CHCl3); (25.6R): 47.3 min, [α]D = −0.80 (c 1.0, CHCl3); L,D-(25.6S): 52.6 min, [α]D = −3.2 (c 0.6, CHCl3). The configurations of D,D-(25.6R) and L,D-(25.6S) forms were determined with a known optical rotation after leading to the corresponding DAP. The (2R,6S) and (25.6R) forms were derived to dimethyl 2-N-(benzylxycarbonyl) 6-N'-t-butoxycarbonyl diaminopimelate to compare with the known optical rotations; (2R,6S)-dimethyl 2-N-(benzylxycarbonyl) 6-N'-t-butoxycarbonyl diaminopimelate: [α]D = +5.0 (c 0.67, CHCl3), lit. [α]D = +5.45 (c 0.5, CHCl3). After the HPLC separation of dibenzyl 2-N-(benzylxycarbonyl) 6-N'-t-butoxycarbonyl diaminopimelate, the protecting groups of the obtained isomers were cleaved by refluxing for 5 h in 6 N HCl (15 M), and then concentrated under reduced pressure. The residues were separately washed with Et2O to give (2R,6R)-DAP, meso-(2R,6S)-DAP, and (2S,6S)-DAP (DD-DAP, meso-DAP, and LL-DAP) as white crystals. Electrospray ionization-mass spectrometry (positive) m/z [M+H]+. The 1H-nuclear magnetic resonance spectroscopic data and optical rotation data were consistent with those reported.

Cells and cell culture

The human oral epithelial cell lines HSC-2 (15) and HO-1-u-1 (16), the human pharyngeal epithelial cell line HEp-2, the human esophageal epithelial cell line TE-1, and the human monocytic cell line THP-1 were obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The human colon adenocarcinoma cell line SW620 (CCL-227) was purchased from the American...
Type Culture Collection. Human epithelial carcinoma of cervix HeLa (JCRB9904), HO-1- u-1, HEp-2, TE-1, SW620, and HeLa cells were grown in RPMI 1640 medium (Nissui Seiyaku) with 10% heat-inactivated FCS (JCRB9904), and human epithelial carcinoma of cervix HeLa cells were grown in RPMI 1640 medium with 1% FCS for 24 h at 37°C, according to the manufacturer’s instructions. The cellular viability of the cells after transfection was >95%, as assessed by a 0.2% trypan blue exclusion test, and morphological character was not changed after transfection. The sequences of target mRNAs used in this study are: TLR2, 5’-AAATCCGGAGGCTGCATATCC-3’/H11032, NOD1, 5’-AATCCGGAGGCTGCATATCC-3’/H11032; and NOD2, 5’-AAGACATCTTCCAGTTACTCC-3’/H11032. siRNA for TLR2, NOD1, and NOD2 were synthesized and purified by Silencer siRNA Construction kit (Ambion).

Flow cytometry
Flow cytometric analyses were performed using a FACScalibur cytometer (BD Biosciences). The cells were stimulated with or without test materials for 24 h at 37°C. After incubation, cells were collected by nonenzymatic dissociation solution and washed in PBS. Cells were stained with anti-PGRP-I mAb (mouse IgG1) (Imgenex), followed by FITC-conjugated goat anti-mouse IgG (BioSource International).

Immunostaining
Epithelial cells were cultured on eight-chamber glass slides (Falcon) until confluent and treated with test materials for 24 h at 37°C in a 5% CO2 incubator and washed three times with PBS. After fixation with 4% paraformaldehyde for 15 min at room temperature, cells were treated with mouse anti-PGRP-I mAb (1/100) for 3 h at room temperature without permeabilizing. For β-defensin II staining, experimental system was performed. Briefly, after fixation, the cells were treated with 0.5% Triton X-100 for 15 min and washed with PBS. Cells were then treated with goat anti-human β-defensin 2 (1/100) Ab for 3 h at room temperature. As a negative control, mouse IgG1 or goat Ig (DakoCytomation) was used. Samples were then washed and incubated with Alexa Fluor 488 goat anti-mouse IgG1 (1/500) or Alexa Fluor 488 rabbit anti-goat IgG (1/500), respectively. Nuclei were visualized by staining with 4′,6-diamino-2-phenylindole (DAPI) in blue (Molecular Probes). Samples were photographed with an AxioCamMR monochrome digital camera mounted on the Zeiss Axiolmager Z1 microscope with the application Zeiss AxioVision 4.0 software (Zeiss).

Statistical method
Statistical significances were determined using ANOVA with the Bonferroni or Dunnnett method.

Results
Up-regulation of the expressions of PGRPs upon stimulation with meso-DAP in oral epithelial cells
To examine the bacterial moiety detected by NOD1, we first used human oral epithelial HSC-2 cells and carefully examined the ability

RNA isolation and real-time PCR
Total cellular RNA was prepared from human oral epithelial cells with Isogen (Nippon Gene) according to the manufacturer’s instructions. Random hexamer-primed reverse transcription was performed using 2.5 µl of total RNA in a 50-µl reaction volume. Real-time PCR was performed with a LightCycler. The specificity of the PCR was confirmed by the molecular weight of the products and a melting curve analysis at each data point. The primers used for PCR had the following sequences: PGRP-L, 5’-ACT GAGGCGTCTGGAGAACC-3’; 5’-GGCTCATGTAATAFCCTTGG-3’; PGRP-1α, 5’-GTTCGCGTCTCCAGGAAACCA GAGAGACAC-3’; PGRP-Iβ, 5’-ATGTCACCCACGCTTCTCT-3’; 5’-CACCCACTGTGTTGGAC-3’; PGRP-S, 5’-CGTGCGTCTGGAAC-3’; TCAG-3’; 5’-GCACTTCCCCGTTCCCTTG-3’; and human GAPDH, 5’-GATCCACCATCITCCCAGGACGC-3’; 5’-CATGAGTCTTTCGAGT ACC-3’. Cycling conditions were as follows: with PGRP-L, 40 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 11 s; with GAPDH, 40 cycles at 95°C for 15 s, 56°C for 15 s and 72°C for 11 s. The results are expressed as relative mRNA accumulation corrected with reference to GAPDH mRNA as an internal standard.

Measurement of cytokines
The cells (10^5 cells per 200 µl) were incubated with or without stimulant in RPMI 1640 medium with 1% FCS for 24 h in 96-well flat-bottom plates (Falcon; BD Discovery Labware). In some experimental systems, cytochalasin D (1 µM) was added directly to the cell culture medium 30 min before stimulation to allow better stimulation by NOD ligands (17). After cultivation, the culture supernatants were collected and the levels of IL-8, MCP-1, IL-6 and TNF-α were determined with ELISA kits (BD Pharmingen). The concentration of cytokines in the supernatants was determined using the SOFTMAX data analysis program (Molecular Probes).

RNA interference
Transfections for targeting endogenous TLR2, NOD1, and NOD2 were conducted using Lipofectamine 2000 (Invitrogen Life Technologies) and short interfering (si) RNA (final concentration, 200 nM) for 24 h at 37°C, according to the manufacturer’s instructions. The cells were transfected with buffer only or siRNA of TLR2, NOD1, or NOD2. Lamin A/C was used as a negative control. After 24 h, total RNA was extracted and RT-PCR was performed using primers for the respective genes. After a 24-h incubation, cells were stimulated with 100 µg/ml meso-DAP, n-Gln, or MDP or 100 ng/ml lipid A. After 8 h, the mRNA expressions of PGRPs were analyzed by real-time PCR. *, p < 0.01 and #, p < 0.01 vs the respective control. Error bars indicate SD. Results are representative of three different experiments.
of PGRP induction. A commercial DAP preparation for itself up-regulated the mRNA expressions of four PGRPs on human oral epithelial cells in culture in dose-dependent manners, whereas L-Lys and D-Gln, both of which are also key amino acids in bacterial PGN, were inactive (Fig. 2A). The commercial DAP is a mixture of stereoisomers; DD-, LL-, and meso-DAP, among which meso-DAP is a PGN component of most Gram-negative and some Gram-positive bacteria such as Mycobacterium, Corynebacterium, and Bacillus, and LL-DAP was isolated from specific bacteria such as Clostridium perfringens and Propionibacterium acnes, whereas DD-DAP has not been isolated from any bacteria so far. Therefore, we chemically synthesized three isoforms of DAP (Fig. 1C). In flow cytometry, meso-DAP induced PGRP-Iβ protein on human oral epithelial cells, whereas DD-DAP and LL-DAP were inactive in this respect (Fig. 2B). A similar finding was also obtained by immunostaining (Fig. 2C); meso-DAP induced PGRP-Iβ expression and LL-DAP slightly induced PGRP-Iβ expression, whereas DD-DAP did not induce PGRP-Iβ expression.

FIGURE 4. Induction of proinflammatory cytokines in epithelial cells triggered by meso-DAP. SW620 cells (A–E) and IFN-γ-treated (1000 IU/ml) HSC-2 cells (F–J) were stimulated with or without indicated dose of test materials for 24 h. J, SW620 cells were preincubated with or without cytochalasin D (1 μM) for 30 min before stimulation to facilitate stimulation by NOD ligands. Then, cells were stimulated with or without the indicated dose of test materials for 24 h. At the concentration used, cytochalasin D treatment for 24 h did not affect cell viability, as assessed by measurement of lactate dehydrogenase release in the culture supernatant (data not shown). IL-8, MCP-1, IL-6, and TNF-α levels in the culture supernatants were determined by ELISA. Data are expressed as mean values ± SD, and significant differences are shown. *, p < 0.01 vs medium alone. The results presented are representative of three different experiments demonstrating similar results.
Specific suppression of PGRP mRNA expression induced by meso-DAP using siRNA targeting NOD1

To clarify the signaling pathway of cellular activation by meso-DAP, we used RNA interference assays targeting NOD1, NOD2, and TLR2 mRNA. The region of the TLR2, NOD1, and NOD2 mRNA targeted by the siRNA is shown in Materials and Methods. We confirmed that TLR2, NOD1, and NOD2 mRNA levels were suppressed by ~80% using specific siRNA in oral epithelial HSC-2 cells from 24 h to 72 h of culture (Fig. 3A) (12). The up-regulated expressions of the mRNAs for PGRPs induced by meso-DAP were inhibited in NOD1-silenced oral epithelial cells, but neither NOD2- nor NOD2-silenced oral epithelial cells (Fig. 3). However, siRNA for NOD1 did not inhibit responses induced by MDP (NOD2 agonist) nor lipid A (TLR4 agonist) in the same cells (Fig. 3). These results indicated that regulation of PGRP mRNA by meso-DAP occurs through NOD1.

Secretion of proinflammatory cytokines upon stimulation with meso-DAP in intestinal epithelial cells and IFN-γ-primed oral epithelial cells

To further clarify cellular activation triggered by meso-DAP, we next examined the effect on production of proinflammatory cytokines in human epithelial cells. As described, some human intestinal epithelial cells such as SW620 produce inflammatory cytokines upon stimulation with bacterial components (11). Human intestinal epithelial SW620 cells exhibited the productions of IL-8, MCP-1, IL-6, and TNF-α upon stimulation with meso-DAP in dose-dependent manners, although its activities were in general less than reference NOD agonists (Fig. 4, A–E). In the assay, LL-DAP exhibited weak activity and DD-DAP were inactive.

Naïve oral epithelial cells did not secrete proinflammatory cytokines in response to bacterial cell surface components (11), whereas IFN-γ-primed oral epithelial cells produced cytokines upon stimulation with bacterial components (18). Therefore, we examined whether synthetic DAP preparations activated IFN-γ-treated oral epithelial cells to secrete IL-8. meso-DAP increased MCP-1, IL-6, and TNF-α as well as IL-8 production in IFN-γ-treated oral epithelial HSC-2 cells (Fig. 4, F–H). LL-DAP exhibited weak activity and DD-DAP was also inactive in the assay (Fig. 4A). However, compared with the ability of iE-DAP, FK156, or FK565, the ability of meso-DAP was weakest, whereas iE-DAP showed the strongest activity (Fig. 4I). In our study, very high concentrations of NOD ligands are required to induce cytokines. Recently, Magalhaes (17) added cytochalasin D (1 μM) to the cell culture medium to allow better stimulation by NOD ligands. It has been well established that cytochalasin D inhibits actin polymerization and allows components to internalize into the cells (19). Therefore, we also examined IL-8-inducing abilities of NOD1 ligands against colonic epithelial SW620 cells in the presence of cytochalasin D. The result showed that stimulation with NOD1 ligands in the presence of cytochalasin D (1 μM) exhibited a several hundred-fold higher...
ability to induce cytokines than stimulation with NOD ligands alone in terms of the amount of compound required to achieve 50% of maximum activity (Fig. 4J).

**Meso-lanthionine as well as meso-DAP induced β-defensin 2 in human epithelial cells**

In consistency with results discussed, meso-DAP induced β-defensin 2 generation in oral epithelial HSC-2 cells, whereas DD-DAP and lL-DAP were inactive in this respect (Fig. 5A). It must be noted here that meso-lanthionine also activated oral epithelial cells to generate β-defensin 2 (Figs. 5A). Oral epithelial cells were not stained with goat IgG, followed by Alexa Fluor 488 (green) (data not shown). In addition, other oral epithelial cell line HO-1-u-1, the pharyngeal epithelial cell line HEp-2, the esophageal epithelial cell line TE-1, colon adenocarcinoma cell line SW620 and epitheloid carcinoma of cervix HeLa also

**FIGURE 6.** Meso-DAP might not provoke cellular activation of human monocytic cells even in the presence of lipid A. OCT-treated THP-1 cells were stimulated with DAP isomers and reference NOD agonists (A–D) or those together with lipid A (F–H) for 24 h at indicated concentrations. IL-8, MCP-1, IL-6, and TNF-α level in the culture supernatants was determined by ELISA and mean values are shown. *, p < 0.01 and #, p < 0.01 vs respective control. Significant (p < 0.05) synergistic effects were detected (F–H) by ANOVA including an interaction term. THP-1 cells were stimulated with 100 μg/ml iE-DAP or meso-DAP test material (E) for 8 h. After incubation, mRNA expressions of PGRP-L, PGRP-Iα, PGRP-Iβ, and PGRP-S were analyzed by real-time PCR. Data are expressed as mean values ± SD, and significant differences are shown. *, p < 0.01 vs medium alone. The results presented are representative of three different experiments demonstrating similar results.
shown). Supernatants were determined by ELISA and the mean values are shown.

In the present study, we first demonstrated that meso-DAP, but not DD- and LL-DAP (Fig. 5). These cells were not stained with goat IgG, followed by Alexa Fluor 488 (green) (data not shown). Among human PGRPs, Wang et al. (23) reported that human PGRP-I is involved in intracellular bacterial killing (24). In this and previous (12) studies, PGRP-Iα and PGRP-Iβ were more markedly up-regulated than PGRP-L or PGRP-S, although the functions of mammalian PGRP-Iα and PGRP-Iβ are not yet clear except for PGNs (Fig. 1). In addition, LL-DAP, which was isolated from specific bacteria such as Clostridium perfringens and Propionibacterium acnes, also had a weak potency on cellular activation, whereas DD-DAP, which has been not isolated from any bacteria so far, was inactive in this respect. Furthermore, we demonstrated that synthetic meso-lanthionine was also recognized by NOD1. Meso-lanthionine is a counterpart to meso-DAP in some Fusobacterium and related species such as Fusobacterium nucleatum (13). In contrast, MDP (MurNAc-L-Ala-D-isoGln) or MurNAc-L-Ala-D-Glu, essential NOD2 sensing structures, are almost ubiquitously present in PGNs from both Gram-positive and Gram-negative bacteria (Fig. 1). Therefore, NOD2 can elicit the broad recognition of bacteria, whereas NOD1 mediates the host response to a specified group of bacteria. Both PGN motifs are naturally occurring degradation products released from bacteria during growth (20) or processed by the host cells in the lysosomal compartment. These findings indicate that NOD2 and NOD1 act as cytosolic pattern recognition receptors that recognize highly conserved PGN structures present in all and large populations of bacteria, respectively. Recent studies have shown that the Drosophila immune system detects Gram-positive and Gram-negative bacteria through the specific recognition of respective PGN motifs such as the meso-DAP-containing motif (21). Thus, selective host recognition of bacteria based on the PGN structure is a ubiquitous system from insects to human. It should be emphasized that NOD1 is a likely special sentinel molecule in the epithelial barrier, allowing intracellular detection of bacteria through recognizing the meso-DAP motif of PGN. In fact, Inohara et al. (22) reported that NOD1 was expressed in multiple tissues and was thought to have an important role, particularly in epithelium. Therefore, NOD1 and NOD2 in cooperation might recognize DAP-type bacteria and induce sufficient protective responses against these bacterial invasions. In this study, MDP, iE-DAP, meso-DAP, FK156, and FK565 are used from the 1 to 100 μg/ml range. Girardin et al. (6, 9, 10) reported significant activity of NOD agonists at the 50 pM/ml, which was a similar concentration to that of lipid A as a TLR4 agonist. It must be noted that Girardin et al. (6, 9, 10) microinjected test materials into cells, or cells were permeabilized by digitonin, whereas we only added the test materials to cell cultures without treatment to enhance permeability to examine responses under physiological conditions in most of the experiments in this study. Therefore, our system was not efficient for putting test materials into the cytoplasm of the cells, and excess amounts of materials may be required for cell activation. In this context, in the presence of cytochalasin D (1 μM) to facilitate stimulation by NOD ligands according to Magalhaes et al. (17), NOD1 ligands, including meso-DAP, exhibited a several hundredfold higher ability to induce IL-8 on human epithelial SW620 cells compared with that after stimulation with NOD1 ligands alone in terms of the amount of compound required to achieve 50% of maximum activity (Fig. 4J). In the system, human monocytic THP-1 cells also respond to meso-DAP, although the cells scarcely respond to meso-DAP in the absence of cytochalasin D (Fig. 7). These findings suggested that NOD1 is capable of recognizing meso-DAP even in monocytic cells, although under the physiological conditions meso-DAP could not be transferred to NOD1 in monocytic cells in contrast to epithelial cells.

Among human PGRPs, Wang et al. (23) reported that human PGRP-L is MurNAc-L-Ala amidase like PGRP-SC1B in Drosophila, both of which digest PGN. Murine PGRP-S was suggested to be involved in intracellular bacterial killing (24). In this and previous (12) studies, PGRP-1α and PGRP-1β were more markedly up-regulated than PGRP-L or PGRP-S, although the functions of mammalian PGRP-1α and PGRP-1β are not yet clear except for the present study, we first demonstrated that meso-DAP itself activates human cells via NOD1. Meso-DAP is a specific amino acid to most Gram-negative and some Gram-positive bacteria, and is a key moiety to make cross-link between stem peptides of these human monocytic cells in the presence of cytochalasin D. OCT-treated THP-1 cells were preincubated with cytochalasin D (1 μM) for 30 min before stimulation to allow better stimulation by NODs ligands. Then, cells were stimulated with DAP isomers and reference NOD agonists for 24 h at the indicated concentrations. IL-8, MCP-1, IL-6, and TNF-α levels in the culture supernatants were determined by ELISA and the mean values are shown. *, p < 0.01 vs medium alone. The results presented are representative of three different experiments demonstrating similar results.

Human monocytic cells scarcely respond to meso-DAP

Then, we examined whether meso-DAP activates human monocytic cells. All of three DAP isomers did not induce IL-8 production in human monocytic THP-1 cells, in which synthetic FK156, FK565, and iE-DAP as reference NOD1 agonists and MDP as an NOD2 agonist showed marked activities to augment production of IL-8, MCP-1, IL-6, and TNF-α (Fig. 6, A–D). In addition, meso-DAP did not induce the expression of PGRPs mRNA on THP-1 cells (Fig. 6E). We recently demonstrated that MDP and DMPs in combination with TLR agonists synergistically induced the production of IL-8 in an NOD2- and NOD1-dependent manner, respectively, in human monocytic THP-1 cells. We further examined possible synergistic IL-8 secretion by meso-DAP in combination with lipid A in THP-1 cells in culture. Meso-DAP did not enhance cellular activation even in combination with lipid A, whereas iE-DAP had a potency for synergistic IL-8 production (Fig. 6, F–H). In contrast, in the presence of cytochalasin D meso-DAP (500 ng/ml) showed slightly but significantly induced the production of IL-6, IL-8, MCP-1, and TNF-α in THP-1 cells (Fig. 7).

Discussion

In the present study, we first demonstrated that meso-DAP itself activates human cells via NOD1. Meso-DAP is a specific amino acid to most Gram-negative and some Gram-positive bacteria, and is a key moiety to make cross-link between stem peptides of these

![FIGURE 7. Meso-DAP provoked cellular activation of human monocytic cells in the presence of cytochalasin D. OCT-treated THP-1 cells were preincubated with cytochalasin D (1 μM) for 30 min before stimulation to allow better stimulation by NODs ligands. Then, cells were stimulated with DAP isomers and reference NOD agonists for 24 h at the indicated concentrations. IL-8, MCP-1, IL-6, and TNF-α level in the culture supernatants were determined by ELISA and the mean values are shown. * p < 0.01 vs medium alone. The results presented are representative of three different experiments demonstrating similar results.](http://www.jimmunol.org/)

![Image of graph showing IL-8, MCP-1, IL-6, and TNF-α levels](http://www.jimmunol.org/)

![Image of graph showing IL-8, MCP-1, IL-6, and TNF-α levels](http://www.jimmunol.org/)
their binding ability to PGN and Gram-positive bacteria. PGRP-Iα and PGRP-Iβ are reported to be highly expressed in the esophagus (25). Both the esophagus and oral cavity are covered by squamous epithelial cells. If PGRP-Iα and PGRP-Iβ functioned actively in the clearance of invasive bacteria, it is of benefit for hosts that PGRP-Iα and PGRP-Iβ expressions are highly up-regulated upon stimulation with bacterial cell surface components in oral epithelial cells, which are the first cells encountered by bacteria in oral mucosa. In contrast, naive oral epithelial cells did not secrete inflammatory cytokines upon stimulation with bacterial components including NOD1 and NOD2 ligands, although IFN-γ-primed oral epithelial cells produced inflammatory cytokines in the same condition. It must be noted that some human intestinal epithelial cell lines such as SW620 and HT29 secreted IL-8 in response to LPS, whereas other human intestinal epithelial cell lines such as T84 and Caco2 cells were completely unresponsive to LPS (26, 27). Furthermore, the responsiveness of human intestinal epithelial SW480 cells was about one-tenth weaker than that of human intestinal epithelial SW620 cells, both of which were isolated from the same person (11). Although proinflammatory cytokine responses of human intestinal epithelial cells to bacterial stimuli have not been examined so far, intestinal epithelial cells produce anti-bacterial factors upon stimulation with bacterial components including NOD1 and NOD2 ligands (28). There is a good possibility that naive epithelial cells in general produce antibacterial factors, but not proinflammatory factors, to prevent bacterial invasion without excessive inflammatory responses, which may lead to tissue destruction.

It is not clear whether the meso-DAP itself actually exhibits bioactivities in vivo. It is interesting that NOD1-deficient mice are generally normal, but they are susceptible to tissue destruction.

References

14. Miyachi, S., T. Moroyama, T. Sakamoto, T. Okamura, and K. Takada. 2005. Chemical synthesis of the muramyl dipeptide itself actually exhibits bioactivities in vivo. It is interesting that NOD1-deficient mice were more susceptible to infection with Helicobacter pylori than wild-type mice (29). In this context, mucosal tissues of NOD-deficient mice are generally normal, but they are susceptible to bacterial infection orally (28). Further studies using NOD1-deficient mice are needed to understand the biological function of NOD1.
15. In addition, bacteria carrying DAP-containing PGN also possess molecules capable of stimulating multiple pattern recognition proteins. Thus, analysis of NOD1 function in the absence and presence of other pattern recognition receptors may be required to unravel the practical roles of NOD1 in host defense against bacteria.

Acknowledgments

We thank Dr. N. Inohara for useful advice and discussion and the late Dr. S. Kotani for encouraging us to carry out this study. We thank D. Mrozek (Medical English Service, Kyoto, Japan) for reviewing this article.

Disclosures

The authors have no financial conflict of interest.