Epidermal Growth Factor Receptor Acts as a Negative Regulator for Bacterium Nontypeable Haemophilus influenzae-induced Toll-like Receptor 2 Expression via an Src-dependent p38 Mitogen-activated Protein Kinase Signaling Pathway

Fumi Mikami, He Gu, Hirofumi Jono, Ali Andalibi, Hirofumi Kai, and Jian-Dong Li
From the Gonda Department of Cell and Molecular Biology, House Ear Institute, and the Department of Otolaryngology, University of Southern California, Los Angeles, California 90057 and the Department of Molecular Medicine, Kumamoto University, Kumamoto 862-0973, Japan

Epidermal growth factor receptor (EGFR) has been shown to play important roles in regulating diverse biological processes, including cell growth, differentiation, apoptosis, adhesion, and migration. Its role in regulating human Toll-like receptors (TLRs), key host defense receptors that recognize invading bacterial pathogens, however, remains unknown. Here we show for the first time that EGFR acts as a negative regulator for TLR2 induction by the bacterium nontypeable Haemophilus influenzae (NTHi) in vitro and in vivo. The negative regulation of TLR2 induction by EGFR is mediated via an Src-MKK3/6-p38 MAP kinase-dependent mechanism. Moreover, direct activation of EGFR signaling by the bacterium NTHi-derived EGFR-like factor appears to be responsible for triggering the downstream Src-MKK3/6-p38 MAPK signaling, which in turn leads to the negative regulation of TLR2 induction. Finally, exogenous EGF increases NTHi invasion of host epithelial cells, thereby demonstrating the biological significance of TLR2 regulation by EGFR signaling. The evidence we provided in the present study may suggest a novel strategy utilized by bacteria to attenuate host defensive and immune response by negatively regulating the expression of host defense receptor TLR2. These studies may bring new insight for fully understanding the important role of EGFR signaling in regulating host defense and immune response by tightly controlling TLR2 induction during bacterial infections.

The epidermal growth factor receptor (EGFR, also known as ErbB-1 or HER) is a type I transmembrane glycoprotein with an extracellular ligand-binding ectodomain and an intracellular cytoplasmic domain (1–4). Ligand binding to a monomeric receptor tyrosine kinase activates the kinase activity by promoting receptor dimerization and auto-phosphorylation on tyrosine residues. The latter serve as docking sites for a variety of signaling effectors, which simultaneously initiate many signaling cascades such as Src-dependent activation of MAPK cascade. The EGFR is widely expressed in mammalian epithelial tissues. Despite its important roles in regulating diverse cellular processes, including proliferation, differentiation, apoptosis, migration, and adhesion, the role of EGFR in regulating human defense response, especially the Toll-like receptor (TLR) (5–7), the key host defense receptor, has yet to be determined. Although originally described as a receptor for epidermal growth factors, recent studies have demonstrated that EGFR also acts as receptors for viruses (8–11). In addition, EGFR has also been shown to be activated by the bacterium Helicobacter pylori via an autocrine-dependent manner (12). However, it still remains unclear whether EGFR can be directly activated by bacteria or bacteria-derived factors via a mechanism independent of autocrine signaling.

In the host innate immune system, the surface epithelial cells are situated at host-environment boundaries and thus act as the first line of host defense against pathogenic bacteria (5, 7, 13). The principal challenge for the host is to detect efficiently the invading pathogen and mount a rapid defensive response. They recognize the invading bacteria by directly interacting with pathogen-associated molecular patterns on a variety of bacteria via TLRs expressed on the host (5, 6). Activation of TLRs, in turn, leads to induction of direct antimicrobial activity that can result in elimination of the invading pathogen before a full adaptive immune response takes effect (14). In addition, activation of TLRs is also a prerequisite for the triggering of acquired immunity (15). TLRs are type I transmembrane receptors with leucine-rich repeats in the extracellular domains and cytoplasmic domains that resemble the mammalian interleukin-1 receptor (5–7). To date, 11 members of the human TLR family have been cloned (6, 7, 16). Of these, TLR2 and TLR4 have been well studied. Whereas TLR4 seems to be mainly involved in Gram-negative bacteria lipopolysaccharide signaling, TLR2 can respond to a variety of Gram-positive products, including peptidoglycan, lipoprotein, lipoteichoic acid, and lipoarabinomannan (5–7, 13, 14). The importance of TLR2 in host defense was further highlighted by the studies from knock-out mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive Staphylococcus aureus (17). Furthermore, our recent study demonstrated that TLR2 also plays a key role in activating host immune and inflammatory response by surface lipoprotein from the Gram-negative bacterium nontypeable Haemophilus influenzae (NTHi), a major cause of otitis media and exacerbation of chronic obstructive pulmonary diseases (COPD) (18–22). Thus, it is clear that TLR2 plays a crucial role in host defense against both Gram-positive and -negative bacteria. Although under physiolog-
Negative Regulation of TLR2 by EGFR

TLR2 is expressed at a low level in epithelial cells, its expression is greatly up-regulated during bacterial infections via an NF-κB-dependent manner (23, 24). The finding that TLR2 is greatly induced from low to high levels during bacterial infection may have several important implications in host defense and immune response against bacteria. First, the very low expression of TLR2 we observed in unstimulated epithelial cells is likely to be an important aspect of TLR2 function because under limiting conditions the cellular responses to pathogen-associated molecular patterns could be more stringently regulated by controlling the amounts of TLR protein produced (25). Second, the increased TLR2 expression will probably contribute to the accelerated immune response by epithelial cells as well as the re sensitization of epithelial cells to invading pathogens. If so, tight regulation of TLR2 expression should be one of the important immune-regulatory mechanisms commonly involved in host defense against many bacterial strains. Despite the extensive studies on the roles of TLR in host defense and immune response, how the expression of TLR during bacterial infections is tightly regulated still remains largely unknown. Given the important role that EGFR plays in mediating physiological cellular responses such as proliferation and differentiation, its role in regulating pathological cellular response such as host defense and immune response also remains poorly understood (1–4). The role of EGFR signaling in regulating TLR, the host defense receptor, is totally unclear.

In the present study, we provide evidence for the first time that EGFR acts as a negative regulator for TLR2 induction by bacterium NTHi via an Src-MKK3/6-p38 MAP kinase-dependent mechanism in vitro and in vivo. Direct activation of EGFR signaling by the NTHi-derived EGFR-like factor appears to be responsible for triggering the downstream Src-MKK3/6-p38 MAPK signaling, which in turn leads to the negative regulation of TLR2 induction. Exogenous EGF increased NTHi invasion of host epithelial cells, thereby providing supportive evidence for the biological significance of TLR2 regulation by EGFR signaling. These findings provide novel insights into the role of EGFR signaling in regulating host defense and immune response by tightly controlling TLR2 induction during bacterial infections.

MATERIALS AND METHODS

Reagents—AG1478, SB203580, PP2, and GM6001 were purchased from Calbiochem. Recombinant human EGF was purchased from R&D Systems. Peptidoglycan was purchased from InvivoGen (San Diego).

Bacterial Strains and Culture Conditions—NTHi strain 12, a clinical isolate, was used in this study (18–24). Bacteria were grown on chocolate agar at 37 °C in an atmosphere of 5% CO2. For making NTHi crude extract, NTHi were harvested from a plate of chocolate agar after overnight incubation and incubated in 30 ml of brain heart infusion broth supplemented with NAD (3.5 μg/ml). After overnight incubation, NTHi were centrifuged at 10,000 × g for 10 min, and the supernatant was discarded. The resulting pellet of NTHi was suspended in 10 ml of phosphate-buffered saline and sonicated. Subsequently, the lysate was collected and stored at −70 °C. Live bacteria of NTHi at an m.o.i. of 100:1 were used to treat the epithelial cells. NTHi lysates (15 μg/ml) were used to treat the cells for all of the other experiments. We chose to use NTHi lysates because of the following reasons. First, NTHi has been shown to be highly fragile and has the tendency to autolyze. Its autolysis can be triggered in vivo under various conditions, including antibiotic treatment. Therefore, using lysates of NTHi represents a common clinical condition in vivo, especially after antibiotic treatment.

Cell Culture—Human cervix epithelial cell line HeLa was maintained as described (18, 23, 24). MDA-MB453, a breast cancer epithelial cell line in which the level of EGFR expression is not detectable with anti-EGFR antibody (catalog number 2232, Cell Signaling Technology), and MDA-MB468, another breast cancer epithelial cell line in which the level of EGFR expression is readily detectable with the same anti-EGFR antibody (American Type Culture Collection), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) (9). All media received additions of 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Real Time Quantitative PCR Analysis of TLR2 and EGF—Total RNA was isolated by using TRIzol reagent (Invitrogen) by following the manufacturer’s instructions. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. Briefly, the reverse transcription reaction was performed for 60 min at 37 °C, followed by 60 min at 42 °C by using oligo(dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix for TLR2 and mTLR2 or SYBR Green Universal Master Mix for EGF. In brief, reactions were performed in duplicate containing 2× Universal Master Mix, 1 μl of template cDNA, 100 nM primers, and 100 nM probe in a final volume of 12.5 μl, and they were analyzed in a 96-well optical reaction plate (Applied Biosystems). Probes for TaqMan included a fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5’ end and labeled with a fluorescent quencher dye, 6-carboxytetramethylrhodamine, on the 3’ end to allow direct detection of the PCR product. Reactions were amplified and quantified by using an ABI 7700 sequence detector and the manufacturer’s corresponding software (Applied Biosystems). Relative quantity of mRNAs was obtained by using the comparative Ct method (for details, see User Bulletin 2 for the ABI PRISM 7700 sequence-detection system) and was normalized by using predeveloped TaqMan assay reagent human cyclophilin as an endogenous control (Applied Biosystems). The primers and probes for TLR2 were as follows: forward primer, 5’-GCC CAG CAA ATT ACC TGT GTG-3’, reverse primer, 5’-AGG CGG ACA TCC TGA ACC T-3’, and TaqMan probe, 5’-FAM-TCC ATC CCA TGT GGC TGG CC-3’. The primers and probes for mTLR2 were as follows: forward primer, 5’-AAG GCA TTA AGT CTC CGG AAT TAT C-3’, reverse primer, 5’-TCA CTT AAG CGA GTA CCT GTC TTT TCT GCT-3’, and TaqMan probe, 5’-FAM-TCC CAA AGT CTA AAG TGC ATC CGC GAC-3’. The primers for EGF were as follows: forward primer, 5’-GCC CCA TTC TCT CCT ATC AGC-3’, and reverse primer 5’-GTC AGC TCC ATT TGG GTG GGT-3’.

Plasmids and Transfections—The expression plasmids of p38α (AF), p38β, (AF), MKK3 (A), MKK6 (A), MKK3 (E), MKK6 (E), EGFR DN, EGFR wild type, Src DN, and Src CA were as described previously (18, 21–24, 26–28). All of the transient transfections were carried out in duplicate using TransIT-LT1 reagent (Mirus, Madison, WI) following the manufacturer’s instructions (18–24). In all co-transfections, an empty vector was used as a control.

RNA-mediated Interference—EGFR small interfering RNA oligonucleotide was purchased from Dharmacon. The siRNA was transfected into HeLa cells using RNAiFect transfection reagent (Qiagen) following the manufacturer’s instructions (29).

Immunoprecipitation—For immunoprecipitation, 500 μl of lysates were incubated for 30 min at 4 °C with control goat IgG antibody and protein A-agarose (Santa Cruz Biotechnology). After centrifugation, EGFR antibody (Santa Cruz Biotechnology) was incubated with supernatant for 1 h at 4 °C, followed by incubation overnight with protein A-agarose. Immunoprecipitates were washed three times with RIPA buffer, resuspended in 2× SDS loading buffer, and separated on 6% SDS-PAGE, followed by Western blot analysis.

Western Blot Analysis—Antibodies against phospho-p38, p38, phospho-MKK3/6, MKK3, EGFR, and phospho-Src (Tyr-416) were pur-
chased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-Tyr and c-Src were purchased from Santa Cruz Biotechnology. Antibody against H-9252-actin was purchased from Sigma. Phosphorylations of p38, MKK3/6, and Src were detected as described and by following the manufacturer’s instructions (18, 21, 24).

EGF Neutralization Assays—For neutralization assay, NTHi lysates were mixed with purified mouse IgG or human EGF antibody (R & D Systems) at the indicated concentrations and incubated for 1 h before addition to cells (12). Cells were treated for 3 h or 15 min and collected for real time quantitative PCR analysis or Western blotting, respectively.

EGFR Neutralization Assays—For neutralization assay (11), cells were incubated for 1 h with purified mouse IgG or human EGFR antibody (R&D Systems) at the indicated concentrations. Cells were then treated with NTHi for 3 h and harvested for further real time quantitative PCR analysis.

Immunodepletion—For immunodepletion, 60 μg of NTHi lysates was incubated with control rabbit IgG antibody and protein A-agarose (Santa Cruz Biotechnology) for 30 min at 4°C. After centrifugation, the supernatants were incubated with or without human EGF antibody (Santa Cruz Biotechnology) for 1 h at 4°C, followed by incubation with protein A-agarose for overnight. Supernatants were then tested for their effects on TLR2 expression.

EGF ELISA Assay—For EGF ELISA assay, ELISA plates were coated with 150 μl of 0.01M citrate buffer and 150 μl of cell conditioned media overnight at 4°C. After washing with 0.05% PBS-T, 200 μl of 10 mg/ml bovine serum albumin was added and incubated at room temperature for 1 h. Following this, 100 μl of human EGF antibody (R&D Systems) diluted in 0.05% PBS-T was added and incubated for 1 h. Next, 100 μl of horseradish peroxidase conjugate anti-mouse antibody was added for 1 h. Finally, 100 μl of TMB substrate buffer (Bio-Rad) was added for 30 min and color-developed in relation to the amount of EGF present. The reaction was stopped by adding 40 μl of 4 M sulfuric acid, and the degree of color that had been generated was determined by measuring the absorbance at 450 nm.

Gentamycin Survival Assay—The number of viable intracellular bacteria was determined by using a standard gentamycin survival assay (41, 42).

FIGURE 1. EGF receptor is a negative regulator for the bacterium NTHi-induced TLR2 expression in vitro and in vivo. A, pretreatment with AG1478 (1, 2 μM), a specific inhibitor for EGFR, enhanced NTHi-induced TLR2 expression at an mRNA level in HeLa cells, as assessed by performing real time quantitative PCR analysis. B, overexpression of a dominant-negative mutant of EGFR-enhanced NTHi-induced TLR2 expression at an mRNA level, whereas overexpression of a wild-type (WT) EGFR attenuated NTHi-induced TLR2 expression at an mRNA level in HeLa cells. C, transfection with EGFR-siRNA (100 nM) enhanced NTHi-induced TLR2 expression at an mRNA level. D, NTHi-induced TLR2 expression at an mRNA level was markedly enhanced in MDA-MB453 (hereafter MB453), a breast cancer epithelial cell line in which the level of EGF expression is not detectable with anti-EGF antibody (catalog number 2232, Cell Signaling Technology), as compared with that in MDA-MB468 (hereafter MB468), another breast cancer epithelial cell line in which the level of EGF expression is readily detectable with the same anti-EGF antibody. E, overexpression of a wild-type EGFR inhibited NTHi-induced TLR2 expression at an mRNA level in MDA-MB453 cells. F, pretreatment with AG1478 (2 μM) enhanced NTHi-induced TLR2 at the protein level in HeLa cells. G, NTHi-induced TLR2 expression at protein level was markedly enhanced in MDA-MB453 cells, as compared with that in MDA-MB468 cells. In experiments described in F and G, cells were treated with or without NTHi for 7 h. Protein lysates were then assessed by Western blotting using anti-hTLR2 antibody (H-175). H, AG1478 greatly enhanced TLR2 induction by NTHi at an mRNA level in the lung of BALB/c mice. Values are the mean ± S.D.; n = 3. CON, control.
42). HeLa cells in 6-well plates were exposed to NTHi at an m.o.i. of 100:1 for 7 h with or without 10 ng/ml of EGF. The monolayers were next washed twice with minimum Eagle's medium. Fresh minimum Eagle’s medium containing 250 μg/ml gentamycin was then added to kill extracellular bacteria. 2 h after gentamycin treatment, the monolayers were washed three times and then lysed by the addition of 1 ml of sterile 1% saponin in PBS. The lysed cells were mixed thoroughly by vigorous pipetting and serially diluted in PBS. The dilutions were plated on prewarmed chocolate agar plate and incubated overnight, and the number of colonies was counted and used to estimate the number of colony-forming units per well. Culture supernatants harvested after gentamycin treatment were also tested to confirm that no viable bacteria remained.

In Vivo Study—BALB/c mice were purchased from Charles River Breeding Laboratories, and 7–8-week-old mice were used in this study. After the trachea was surgically exposed by middle line incision in the skin, NTHi was directly injected into the trachea. Lung tissues were collected and then stored at −80°C. In experiments using chemical inhibitors, AG1478 (1 mg/mouse; 50 mg/kg) or PP2 (0.2 mg/mouse; 10 mg/kg) or an equal volume of control vehicle was given via an intraperitoneal route for 30 min before the inoculation of NTHi into the trachea. Total RNA was isolated from the frozen tissue using TRIzol reagent. Three mice were used for each inoculation group. The House Ear Institute Institution’s Animal Care and Use Committee approved all of the animal protocols used in this study.

RESULTS

EGF Receptor Is a Negative Regulator for Bacterium NTHi-induced TLR2 Expression in Vitro and in Vivo—EGFR is a pleiotropic signaler that has been shown to be involved in cell growth, differentiation, apo-
EGFR is negatively involved in NTHi-induced TLR2 transcription in vitro and in vivo. As shown in Fig. 1, G was much higher in MB453 as compared with MB468 cells (Fig. 1, induction at the protein level, and TLR2 induction at the protein level expression at the mRNA level as assessed by real time quantitative PCR, whereas overexpression of a wild-type EGFR reduced it, thereby further supporting the negative involvement of EGFR (Fig. 1B). We next confirmed the negative role of EGFR in TLR2 induction by using an siRNA approach. We first verified the efficiency of EGFR-specific siRNA in reducing EGFR expression in HeLa cells. As expected, the EGFR protein was markedly reduced by EGFR siRNA (Fig. 1C, upper panel). We then assessed the effect of EGFR siRNA on TLR2 induction by NTHi. As shown in Fig. 1C (lower panel), EGFR knockdown by EGFR siRNA greatly enhanced NTHi-induced TLR2 expression. To confirm further whether EGFR is a negative regulator for TLR2 induction, we examined TLR2 induction by NTHi in MDA-MB453 (hereafter MB453), a breast cancer epithelial cell line in which the level of EGFR expression is greatly enhanced by NTHi-induced TLR2 expression at an mRNA level, whereas overexpression of a constitutively active form of Src inhibited NTHi-induced TLR2 expression at an mRNA level in HeLa cells. D, PP2 also greatly enhanced TLR2 induction by NTHi at an mRNA level in the lungs of BALB/c mice. Values are the mean ± S.D.; n = 3. CON, control.

EGFR Is Negatively Involved in NTHi-induced TLR2 Transcription via an MKK3/6-p38 α/β MAP Kinase-dependent Mechanism—We next sought to determine how EGFR is negatively involved in TLR2 induction by NTHi. On the basis that p38 MAPK has been shown to act downstream of EGFR (30–32) and we previously showed that the MKK3/6-p38 MAPK signaling pathway is negatively involved in TLR2 induction (23–24), it is logical that EGFR is negatively involved in TLR2 induction by acting upstream of the MKK3/6-p38 pathways. We first confirmed that MKK3/6-p38 is indeed negatively involved in TLR2 induction. As shown in Fig. 2A, perturbing MKK3/6-p38 α/β signaling using either the chemical inhibitor for p38, SB203580, or overexpressing dominant-negative mutant forms enhanced TLR2 induction by NTHi, whereas overexpressing a constitutively active form of MKK3 (MKK3 CA) or MKK6 (MKK6 CA) reduced TLR2 up-regulation (Fig. 2A). We next sought to determine whether EGFR acts as a negative regulator for TLR2 induction via MKK3/6-p38 signaling. Our hypothesis was first supported by the result shown in Fig. 2B that AG1478, the inhibitor for EGFR, no longer enhanced NTHi-induced TLR2 up-regulation if the cells were already pretreated with SB203580, the inhibitor for p38, suggesting that AG1478 and SB203580 may target the same p38 MAPK signaling pathway. Consistent with this result, activation of p38 signaling by overexpressing the constitutively active form of either MKK3 or MKK6 attenuated TLR2 induction by NTHi in MB453 cells (Fig. 2C). To confirm that EGFR does act upstream of the MKK3/6-p38 pathway, we then evaluated the effects of inhibiting EGFR signaling on NTHi-induced activation of MKK3/6 and p38 by performing Western blot analysis using antibodies against phosphorylated MKK3/6 or p38. As shown in Fig. 2, D–F, NTHI-induced activation of MKK3/6 and p38 was greatly inhibited by inhibiting EGFR signaling using multiple approaches, including using the EGFR inhibitor AG1478 (Fig. 2D), overexpressing the dominant-negative mutant form of EGFR (Fig. 2E), and knockdown of EGFR expression using the EGFR siRNA approach (Fig. 2F). Moreover, NTHI-induced activation of MKK3/6 and p38 was observed only in MB468 but not in MB453 cells (Fig. 2G). Their responsiveness to NTHi could be rescued by overexpression of wild-type of EGFR in MB453 cells, similarly to the response of MB468 cells (Fig. 2H).

Consistent with these results, exogenous EGF did induce p38 activation in these cells (Fig. 2H). We next sought to determine whether EGFR acts as a negative regulator for TLR2 induction via MKK3/6-p38 signaling. Our hypothesis was first supported by the result shown in Fig. 2B that AG1478, the inhibitor for EGFR, no longer enhanced NTHi-induced TLR2 up-regulation if the cells were already pretreated with SB203580, the inhibitor for p38, suggesting that AG1478 and SB203580 may target the same p38 MAPK signaling pathway. Consistent with this result, activation of p38 signaling by overexpressing the constitutively active form of either MKK3 or MKK6 attenuated TLR2 induction by NTHi in MB453 cells (Fig. 2C). To confirm that EGFR does act upstream of the MKK3/6-p38 pathway, we then evaluated the effects of inhibiting EGFR signaling on NTHi-induced activation of MKK3/6 and p38 by performing Western blot analysis using antibodies against phosphorylated MKK3/6 or p38. As shown in Fig. 2, D–F, NTHI-induced activation of MKK3/6 and p38 was greatly inhibited by inhibiting EGFR signaling using multiple approaches, including using the EGFR inhibitor AG1478 (Fig. 2D), overexpressing the dominant-negative mutant form of EGFR (Fig. 2E), and knockdown of EGFR expression using the EGFR siRNA approach (Fig. 2F). Moreover, NTHI-induced activation of MKK3/6 and p38 was observed only in MB468 but not in MB453 cells (Fig. 2G). Their responsiveness to NTHi could be rescued by overexpression of wild-type of EGFR in MB453 cells, similarly to the response of MB468 cells (Fig. 2H). Consistent with these results, exogenous EGF did induce p38 activation in these cells (Fig. 2H). Collectively, these data demonstrate that EGFR

Negative Regulation of TLR2 by EGFR
indeed acts as a negative regulator for TLR2 induction by acting upstream of the MKK3/6-p38 signaling pathway. Because TLR2 itself also activates p38, we sought to determine further the effect of the p38 inhibitor on TLR2 expression during signaling by TLR2 ligands. Most interestingly, blocking p38 signaling using SB203580 also enhanced TLR2 induction by peptidoglycan, a known ligand for TLR2 (Fig. 2J) (5–7). Thus, these data suggest that once up-regulated, TLR2 may also contribute to activation of p38, which in turn leads to the negative regulation of TLR2 itself. However, given the very low expression of TLR2 in human epithelial cells under unstimulated conditions, the contribution from TLR2 in p38 activation during the initial phase of NTHi bacterial infection, if any, may be minimal. Nonetheless, these data do suggest the existence of an interesting TLR2-p38 signaling-dependent negative feedback loop on TLR2 itself during late phases of NTHi infections.

Src Acts as the Intermediate Signaling Molecule between EGFR and MKK3/6-p38 MAPK in Negative Regulation of NTHi-induced TLR2 Transcription—Having identified the negative role of EGFR-dependent MKK3/6-p38 pathway in mediating NTHi-induced TLR2 expression, it is still unknown which intermediate signaling molecule links EGFR to MKK3/6-p38. Among all known signaling molecules involved in mediating signals from EGFR to MKK3/6-p38, the nonreceptor tyrosine kinase Src appears to play an important role in linking EGFR signaling to activation of p38 in a variety of cellular stress responses (30–35). We therefore sought to determine whether Src is also negatively involved in NTHi-induced TLR2 expression. We first assessed whether Src is activated by NTHi. As shown in Fig. 3A, Src phosphorylation was enhanced by NTHi in MDA-MB468 cells. Overexpression of a constitutively active form of Src (Src CA) inhibited TLR2 induction by NTHi (Fig. 3C). Furthermore, overexpression of a wild-type (WT) Src enhanced NTHi-induced expression of TLR2 in MDA-MB453 cells, thereby confirming that the Src signaling pathway also acts as a negative regulator for NTHi-induced TLR2 transcription. We concluded from these data that Src is also a negative regulator for NTHi-induced TLR2 expression in vitro and in vivo.

We next sought to determine whether Src acts downstream of EGFR signaling by assessing the effects of inhibiting EGFR signaling of PP2, a specific Src inhibitor, and overexpression of a dominant-negative mutant form of Src. As shown in Fig. 3B, exposure of HeLa cells to PP2 enhanced TLR2 induction by NTHi. Overexpressing a dominant-negative mutant of Src (Src DN) enhanced and overexpressing a constitutively active form of Src (Src CA) inhibited TLR2 induction by NTHi in vivo, confirming that Src is also a negative regulator for NTHi-induced TLR2 expression in vitro and in vivo.
on Src phosphorylation. NTHi-induced activation of Src was greatly inhibited by inhibiting EGFR signaling using multiple approaches, including using the EGFR inhibitor AG1478 (Fig. 4A), overexpressing the dominant-negative mutant form of EGFR (Fig. 4B), and knockdown of EGFR expression using the EGFR siRNA approach (Fig. 4C). Moreover, NTHi-induced activation of Src was observed only in MB468 but not in MB453 cells (Fig. 4D). Their responsiveness to NTHi could be rescued by overexpression of the wild-type EGFR in MB453 cells, similarly to the response of MB468 cells (Fig. 4E). Finally, activation of Src signaling by overexpression of the constitutively active form of Src (Src CA) inhibited TLR2 induction by NTHi in MB453 cells (Fig. 4F). Consistent with these results, exogenous EGF did induce Src activation (Fig. 4G). Together, our data suggest that Src indeed acts downstream of EGFR in mediating the negative regulation of TLR2 induction by NTHi.

To determine further whether Src also acts upstream of the MKK3/6-p38 signaling pathway, we also examined the effects of inhibiting Src signaling on the NTHi-induced activation of MKK3/6 and p38. As shown in Fig. 5, activation of both MKK3/6 and p38 by NTHi was greatly inhibited by treatment of HeLa cells with PP2, the Src inhibitor (Fig. 5A), and overexpressing a dominant-negative mutant form of Src (Fig. 5B), thereby suggesting that Src indeed acts upstream of the MKK3/6-p38 MAPK signaling pathway.

**NTHi Activates EGFR Signaling Likely via an Autocrine-independent Mechanism**—Although we have demonstrated that activation of EGFR signaling pathway is negatively involved in NTHi-induced TLR2 expression, it is still unclear whether EGFR signaling is activated directly by NTHi-derived EGF-like factor or indirectly by NTHi-induced EGF autocrine signaling. We initially determined how early the NTHi-induced phosphorylation of EGFR could be detected. As shown in Fig. 6A, the NTHi-induced EGFR phosphorylation became evident as early as 1 min, similarly to that induced by EGF. Given such an early phosphorylation of EGFR, it is likely that the early phosphorylation of EGFR might occur as a result of direct activation of EGFR signaling by NTHi, rather than by autocrine signaling.
than NTHi-induced EGF autocrine signaling. We therefore sought to determine whether NTHi-derived EGF-like factor is responsible for the activation of EGFR signaling. We first validated the efficiency of EGF neutralization antibody in neutralizing EGF-induced down-regulation of TLR2 transcription. As shown in Fig. 6B, EGF-induced down-regulation of TLR2 was neutralized by EGF neutralization antibody but not the control antibody. A greatly enhanced TLR2 induction was observed in cells exposed to NTHi lysate that was pretreated with the same EGF neutralization antibody but not the control antibody (Fig. 6C). Likewise, pretreatment of NTHi lysate with EGF neutralization antibody also attenuated its ability in inducing activation of the downstream Src-MKK3/6-p38 signaling pathway as compared with the control antibody (Fig. 6D). Together, these results suggest that direct activation of EGFR signaling by the NTHi-derived EGF-like factor is negatively involved in NTHi-induced TLR2 transcription. Consistent with this finding, pretreatment of the cells with the EGFR ligand binding domain neutralizing antibody, but not the control antibody, enhanced TLR2 induction by NTHi (Fig. 6E). Moreover, NTHi did not up-regulate EGF expression at the mRNA level either in HeLa or MB468 cells (Fig. 6F). To determine whether ectodomain shedding of transmembrane EGF family precursors, including heparin-binding EGF, plays a role in activating EGFR signaling in NTHi-induced TLR2 expression, we then assessed the effect on NTHi-induced TLR2 expression of GM6001, a specific matrix metalloproteinase inhibitor that was routinely used to inhibit ectodomain shedding of transmembrane EGF family precursors (39–40). As shown in Fig. 6G, treatment of cells with GM6001 did not affect TLR2 induction by NTHi, thereby precluding the involvement of metalloproteinase-dependent shedding of EGF precursors in TLR2 induction. Consistent with these findings, NTHi did not induce any detectable increase in EGF in the conditioned media of the epithelial cells as evidenced by ELISA experiments (Fig. 6H). Collectively, these data suggest that NTHi-induced TLR2 transcription is negatively regulated by the EGFR signaling pathway likely via a mechanism independent of EGF autocrine signaling. To determine further whether EGF-like molecules from NTHi may be involved in activating EGFR signaling, we next assessed the effect on TLR2 induction of NTHi lysates in which EGF-like factors were immunodepleted by using an EGF antibody. As shown in Fig. 6I, TLR2 induction was enhanced in cells treated with NTHi lysates in which EGF-like factors were immunodepleted by using an EGF antibody in comparison with the cells treated with NTHi lysates that were treated with control antibody, thereby suggesting that EGF-like factors from NTHi are involved in activating EGFR, which in turn leads to the negative regulation of TLR2 induction. Future experiments are needed to further purify and identify these factors in NTHi lysates using biochemical and genetic approaches.

It should also be noted that in all the experiments described above, NTHi lysates were used. We chose to use NTHi lysates because of the following reasons. First, NTHi has been shown to be highly fragile and has the tendency to autolysate. Its autolysis can be triggered in vivo under various conditions, including antibiotic treatment. Therefore, using lysates of NTHi represents a common clinical condition in vivo, especially after antibiotic treatment. However, given the fact that intact live bacteria do exist at the initial phase of NTHi infections, it is thus logical to also test whether the same modulation of TLR2 can be accomplished by adding intact live bacteria to the cells even if autolysis does occur. Most interestingly, as shown in Fig. 6J, live bacteria did induce TLR2 expression at a moderate level in comparison with NTHi lysates, and further treatment of the live bacteria with antibiotics induced TLR2 expression at a level comparable with that induced by NTHi lysates.

Thus our data suggest that TLR2 induction by NTHi occurs during both early and late phases of NTHi infections.

**Down-regulation of TLR2 by EGFR Signaling Contributes to Increased Bacterial Invasion of Epithelial Cells**—By having demonstrated that EGFR-Src-p38 MAPK pathway acts as a negative regulator for NTHi-induced TLR2 expression, the biological significance of the negative regulation of TLR2 expression by EGFR signaling has yet to be determined. On the basis that TLR2 is involved in host defense response, it is logical to evaluate whether down-regulation of TLR2 by EGFR signaling contributes to increased NTHi invasion of host cells. As shown in Fig. 7, exogenous EGF increased NTHi invasion of host epithelial cells, thereby providing supportive evidence for the biological significance of TLR2 regulation by EGFR signaling.
NEGATIVE REGULATION OF TLR2 BY EGFR

EGFR signaling has been shown to play an important role in regulating cell growth, differentiation, apoptosis, adhesion, and migration (1–4). Its role in regulating human TLR in host defense response, however, remains unknown. In the present studies, we provided direct evidence for the negative involvement of EGFR signaling in mediating bacterium-induced TLR2 transcription both in vitro and in vivo (Fig. 8). We also showed that EGFR acts as a negative regulator for TLR2 induction by acting upstream of the Src-MKK3/6-p38 signaling pathway. Moreover, NTHi activates EGFR signaling likely via an autocrine-independent mechanism. Finally, exogenous EGF increased NTHi invasion of host epithelial cells, thereby demonstrating the biological significance of TLR2 regulation by EGFR signaling. Thus, these observations bring novel insight for fully understanding the important role of EGFR signaling in epithelial cell defense and immune response against invading bacteria by negatively regulating the expression of human TLR.

One important finding in the present study is that EGFR, a growth factor receptor, acts as a negative regulator for TLR2, a major host receptor for bacterial pathogen. TLRs play an important role in recognizing the invading microbial pathogens, including bacteria and viruses (5–7). To date, 11 members of the TLR family have been identified in mammals (6, 7, 16). Of these, TLR2 has been shown recently to act as a cellular receptor for NTHi, a major bacterial pathogen causing the exacerbation of COPD and otitis media (5–7, 13, 14). Our finding that EGFR signaling negatively regulates TLR2 induction by bacteria may have several important implications in host defense and immune response in the molecular pathogenesis of bacterial infections. First, given the fact that stimulation of TLR causes immediate host defensive responses, including the production of an array of antimicrobial peptides and cytokines, negative regulation of TLR2 induction by EGFR signaling may be one of the efficient mechanisms utilized by bacteria to attenuate host defensive response against the invading bacteria themselves. Recently, evidence has emerged that pathogenic bacteria have developed multiple strategies to subvert the host signaling pathways involved in host defensive response during their interactions with their hosts (36). One such an example is the disruption of host MAPK and NF-kB signaling by the Yersinia effector YopJ, a ubiquitin-like protein protease (37). The evidence we provided in the present study may represent a novel strategy utilized by bacteria to attenuate host defensive and immune response by negatively regulating the expression of host defense receptor TLR2. Thus the EGFR-mediated negative regulation of TLR2 induction will lead to the attenuation of host defensive response, thereby favoring the survival of the invading bacterial pathogen. Indeed, this notion was fully supported by the experimental evidence shown in Fig. 7 that down-regulation of TLR2 by EGFR signaling does contribute to increased bacterial invasion of epithelial cells. Second, considering that overactive inflammatory and immune response is detrimental to the host, the EGFR-mediated negative regulation of TLR2 induction may also contribute to the prevention of host tissue damage during bacterial infections, thus favoring the host. Nonetheless, the EGFR-mediated negative regulation of TLR2 induction via an Src-MKK3/6-p38 pathway, together with the TLR-mediated positive regulation of TLR2 transcription via an IKK-1/2δIκBα-NF-κB pathway, may be essential for ensuring the tight control of TLR2 induction during bacterial infections.

Another interesting finding in this study is that EGFR appears to act as a cellular receptor for the EGF-like factors from bacterial pathogen NTHi. This result, although rather unexpected, may provide a novel insight into the role of EGFR in the molecular pathogenesis of bacterial infections. In view of the biological actions of EGFR, significant progress has been made toward fully understanding the role of EGFR in regulating cell proliferation, differentiation, tumorigenesis, and apoptosis. Despite recent studies showing that EGFR may act as a cellular receptor for human virus or mediating virus-induced host cell signaling (9–11), little is known about the role of EGFR in acting as a receptor for bacterial pathogens. Although the bacterium H. pylori has been shown to transactivate EGFR via activation of the endogenous ligand heparin-binding EGF (12), there has been no report showing that EGFR may directly act as a receptor for bacterium-derived EGF-like ligand. Our studies demonstrate for the first time that EGF-like factors from NTHi, a major human bacterial pathogen, may activate EGFR signaling likely via an EGF autocrine-independent mechanism. Several lines of evidence support this notion. First, the NTHi-induced EGFR phosphorylation occurred at as early as 1 min, similarly to that induced by EGF. Given such a rapid activation of EGFR, it is likely that the early phosphorylation of EGFR might occur as a result of direct activation of EGFR signaling by NTHi, rather than NTHi-induced EGF-autocrine signaling (Fig. 6A). Second, pretreatment of NTHi lysate with EGF-neutralizing antibody markedly attenuated its ability in inducing activation of the downstream Src-MKK3/6-p38 signaling pathway, a negative regulator for TLR2 induction, as compared with the control antibody, thereby suggesting that the NTHi-derived EGF-like factor is likely responsible for the activation of EGFR signaling (Fig. 6, B–D). Indeed, when EGF-like factors were immunodepleted from NTHi lysates, TLR2 induction was enhanced by the immunodepleted NTHi lysates (Fig. 6G), thus providing direct evidence for the existence of EGF-like factors in NTHi lysates. Third, pretreatment of the cells with the EGFR ligand binding domain neutralizing antibody, but not the control antibody, enhanced TLR2 induction by NTHi, indicating the requirement of the ligand binding domain of EGFR in mediating the NTHi-induced host response (Fig. 6E). Finally, NTHi did not up-regulate EGF at both mRNA and protein levels and also failed to induce the release of preformed EGF (Fig. 6, F–H). Collectively, these data suggest that NTHi-induced TLR2 transcription is negatively regulated by EGFR signaling pathway via a mechanism independent of EGF autocrine signaling. The molecular identity of NTHi-derived EGF-like factor should be further investigated.

Acknowledgments—We are grateful to Drs. J. Han and C. Basbaum for kindly providing various reagents. We thank the members of Dr. Kai’s laboratory (Graduate School of Pharmaceutical Sciences, Kumamoto University) for stimulating scientific discussions.

REFERENCES

1. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) EMBO J. 19, 3159–3167
2. Yarden, Y., and Sliwkowski, M. X. (2001) Nat. Rev. Mol. Cell. Biol. 2, 127–137
3. Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P., Ward, C. W., and Burgess, A. W. (2003) Exp. Cell Res. 284, 31–53
4. Holbro, T., and Hynes, N. E. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 195–217
5. Aderem, A., and Ulevitch, R. J. (2000) Nature 406, 782–787
6. Akira, S., and Takeda, K. (2004) Nat. Rev. Immunol. 4, 499–511
7. Kopp, E., and Medzhitov, R. (2003) Curr. Opin. Immunol. 15, 396–401
8. Miller, W. E., and Raab-Traub, N. (1999) Trends Microbiol. 7, 453–458
9. Wang, X., Huang, S. M., Chiu, M. L., Raab-Traub, N., and Huang, E. S. (2003) Nature 424, 456–461
10. Compton, T. (2004) Trends Cell Biol. 14, 5–8
11. Monick, M. M., Cameron, K., Staber, J., Powers, L. S., Yarovinsky, T. O., Koland, J. G., and Hunninghake, G. W. (2003) J. Biol. Chem. 278, 2147–2158
12. Keates, S., Sougourolou, S., Keates, A. C., Zhao, D., Peck, R. M., Jr., Shaw, L. M., and Tully, C. P. (2003) J. Biol. Chem. 278, 48127–48134
13. Medzhitov, R., and Janeway, C., Jr. (2000) Trends Microbiol. 8, 452–456
14. Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M. T., Engele, M., Sieling, P. A., Barnes, P. F., Röllinghoff, M., Bölscke, P. L., Wagner, M., Akira, S., Norgard, M. V., Belisle, J. T., Godowski, P. J., Bloom, B. R., and Modlin, R. L. (2001) Science 291,
Negative Regulation of TLR2 by EGFR

1544–1547

15. Iwasaki, A., and Medzhitov, R. (2004) Nat. Immun. 5, 987–995

16. Zhang, D., Zhang, G., Hayden, M. S., Greenblatt, M. B., Bussey, C., Flavell, R. A., and Ghosh, S. (2004) Science 303, 1522–1526

17. Takeuchi, O., Hoshino, K., and Akira, S. (2000) J. Immunol. 165, 5392–5396

18. Shuto, T., Xu, H., Wang, B., Han, J., Kai, H., Gu, X. X., Murphy, T., Lim, D. J., and Li, J. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8774–8779

19. Jono, H., Shuto, T., Xu, H., Kai, H., Lim, D. J., Gum, J. R., Jr., Kim, Y. S., Yamaoka, S., Feng, X. H., and Li, J. D. (2002) J. Biol. Chem. 277, 45547–45557

20. Li, J. D. (2003) J. Pharmacol. Sci. 91, 1–7

21. Jono, H., Xu, H., Kai, H., Lim, D. J., Lim, Y. S., Feng, X. H., and Li, J. D. (2003) J. Biol. Chem. 278, 27811–27819

22. Chen, R., Lim, J. H., Jono, H., Gu, X. X., Kim, Y. S., Basbaum, C. B., Murphy, T. F., and Li, J. D. (2004) Biochem. Biophys. Res. Commun. 324, 1087–1094

23. Shuto, T., Imasato, A., Jono, H., Sakai, A., Xu, H., Watanabe, T., Rixter, D. D., Kai, H., Andalibi, A., Lethenic, F., Guan, Y. L., Han, J., Cato, A. C., Lim, D. J., Akira, S., and Li, J. D. (2002) J. Biol. Chem. 277, 17263–17270

24. Imasato, A., Desbois-Mouthon, C., Han, J., Kai, H., Cato, A. C., Akira, S., and Li, J. D. (2002) J. Biol. Chem. 277, 47444–47450

25. Vainchenker, A., Mazzoni, A., Spitzer, J. H., Wyllie, D. H., Dower, S. K., and Segal, D. M. (2001) J. Immunol. 166, 249–255

26. Wang, B.,Lim, D. J., Han, J.,Kim, Y. S., Basbaum, C. B., and Li, J. D. (2002) J. Biol. Chem. 277, 949–957

27. Lernjakbar, H., and Basbaum, C. (2002) Nat. Med. 8, 41–46

28. Li, J. D., Feng, W., Gallup, M., Kim, J. H., Gum, J., Kim, Y., and Basbaum, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5718–5723

29. Jono, H., Lim, J. H., Chen, L. F., Xu, H., Trompouki, E., Pan, Z. K., Mosialos, G., and Li, J. D. (2004) J. Biol. Chem. 279, 36171–36174

30. Ouwens, D. M., de Ruiter, N. D., van der Zon, G. C., Carter, A. P., Schouten, J., and van der Burg, C., Kooistra, K., Bos, J. L., Maassen, J. A., and van Dam, H. (2002) EMBO J. 21, 3782–3793

31. Keely, S. J., and Barrett, K. E. (2003) Am. J. Physiol. 284, C339–C348

32. Frey, M. R., Golovin, A., and Polk, D. B. (2004) J. Biol. Chem. 279, 44513–44521

33. Thomas, S. M., and Brugge, J. S. (1997) Annu. Rev. Cell Dev. Biol. 13, 513–609

34. Martin, G. S. (2001) Nat. Rev. Mol. Cell. Biol. 2, 467–475

35. Bromann, P. A., Korkaya, H., and Courtneidge, S. A. (2004) Oncogene 23, 7957–7968

36. Boyer, L., and Lemichez, E. (2004) Nat. Rev. Microbiol. 2, 779–788

37. Orth, K., Xu, Z., Mudgett, M. R., Bao, Z. Q., Palmer, L. E., Bliska, J. B., Mangel, W. F., Staskawicz, B., and Dixon, J. E. (2000) Science 290, 1594–1597

38. Wang, X., Huang, D. Y., Huang, S. M., and Huang, E. S. (2005) Nat. Med. 11, 515–521

39. Blobel, C. P. (2005) Nat. Rev. Mol. Cell. Biol. 6, 32–43

40. Tschumperlin, D. J., Dai, G., Maly, I. V., Kikuchi, T., Laiho, L. H., McVittie, A. K., Haley, K. J., Lilly, C. M., So, P. T., Lauffenburger, D. A., Kamm, R. D., and Drazin, J. M. (2004) Nature 429, 83–86

41. Swords, W. E., Buscher, B. A., Ver Steeghl, K., Preston, A., Nichols, W. A., Weiser, J. N., Gibson, B. W., and Apicella, M. A. (2000) Mol. Microbiol. 37, 13–27

42. Swords, W. E., Ketterer, M. R., Shao, J., Campbell, C. A., Weiser, J. N., and Apicella, M. A. (2001) Cell. Microbiol. 3, 525–536
Epidermal Growth Factor Receptor Acts as a Negative Regulator for Bacterium Nontypeable *Haemophilus influenzae*-induced Toll-like Receptor 2 Expression via an Src-dependent p38 Mitogen-activated Protein Kinase Signaling Pathway

Fumi Mikami, He Gu, Hirofumi Jono, Ali Andalibi, Hirofumi Kai and Jian-Dong Li

*J. Biol. Chem.* 2005, 280:36185-36194.
doi: 10.1074/jbc.M503941200 originally published online August 22, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503941200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 18 of which can be accessed free at http://www.jbc.org/content/280/43/36185.full.html#ref-list-1