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Lipopolysaccharide initiates a bypass feedback loop of epidermal growth factor receptor signaling by HPS70-induced COX-2 in H22 hepatocarcinoma cells

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Abstract. LPS can induce TACE upregulation via signaling from TLR4-derived EGFR activation in tumor cells. The regulation and activity of TACE have been investigated with the observation that gene expression is upregulated in response to LPS followed by EGFR activation, however, the process remains poorly understood. In this study, we examined the effects of LPS on H22 hepatocarcinoma cells that displayed constitutively active TLR4 expression. Upon TLR4 shRNA transfection into H22 cells, HSP70 expression significantly increased. However, LPS induced early phosphorylation of EGFR in H22 cells, which reached maximum levels within 30 min. Inhibition of TLR4 in H22 cells resulted in a significant rise in both EGFR phosphorylation and TACE upregulation 24 h after exposure to LPS. Exogenous HSP70 also induced rapid phosphorylation of EGFR, upregulated the expression of COX-2 via a signaling pathway that involved TACE-dependent TGF-α release. Furthermore, inhibition of EGFR activation and reduction of COX-2 expression by COX-2 inhibitor prevented HSP70-induced cell invasion *in vitro*. These findings demonstrate that the biological importance of HSP70/COX-2 is crucial to the second, but not the first, phase of EGFR phosphorylation in tumor cells. The growth of tumor cells by inserting shRNA plasmid TLR4 combination with COX-2 inhibitor could be effectively reduced in LPS stimulation. We concluded that LPS triggered a bypass feedback loop of EGFR activation and involved HSP70/COX-2 in H22 cells by inhibition of TLR4 and that EGFR phosphorylation is implicated in tumor growth by LPS stimulation.

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Introduction

Chronic inflammation has long been associated with increased incidence of malignancy, and similarities in regulatory mechanisms have been suggested for more than a century. Additionally, supporting tumor cells evade antitumor immunity and the infiltration of innate immune cells, such as macrophages and neutrophils, into tumors promotes tumor development by various mechanisms including increased matrix metalloprotease activities, angiogenesis and vasculature density. Although chronic inflammation exhibits a spectrum of malignant potential behavior similar to most tumors, the molecular mechanisms of tumor progression, growth and invasion have not been fully elucidated. Studies on these mechanisms should provide opportunities to design new molecular therapeutic targets.

LPS is a major integral component of the outer membrane of gram-negative bacteria and is one of the most potent stimuli of inflammation. Chronic inflammation promotes neoplastic cell growth, may contribute to neovascularization (1), and is proposed to be an initiating factor in many tumors (2). LPS induces a ligand for TLR4-dependent EGFR phosphorylation, which triggers the MAPK signaling to the nucleus that subsequently causes TACE production. The proposed surface-signaling pathway responsible for the initial activation of EGFR is based on its demonstration in tumor cells (3).

Though initially Hsp70 was thought to be a typical intracellular protein, recent studies demonstrated that Hsp70 is being released into the blood or the conditioned medium of cultured cells under stress conditions (4-6). Exogenously added purified Hsp70 was shown to induce signal transduction that engages lipopolysaccharide receptor CD14 in monocytes and macrophages (7). Moreover, HSP70 resulting in the upregulation of cyclooxygenase-2 (COX-2) expression and EGFR activity (6,8)

that was accompanied by an increase in tumor cell malignancy. TACE proteolytic shedding of EGFR-binding pro-ligands was a key component for the progression of malignancy in tumors characterized by aberrant EGFR activity (9-11). Despite evident communication between the two signaling pathways, the precise mechanism(s) underlying this communication and the pathophysiological implications remain poorly understood.

In the present study, we investigated LPS initiating the signaling cascade that led to EGFR activation, which subsequently induced HPS70-dependent TACE production in TLR-4 inhibition, resulting in a second phase of EGFR activation, modulation of the tumor progression process, and reinforcing the LPS-induced signaling (a bypass feedback loop). The discovery of a novel feedback loop and the finding that this feedback loop is important for cell invasion in response to an inflammatory stimulus (LPS) suggest that this feedback signaling pathway may play an important role in the pathophysiology of carcinogenesis associated with inflammation.

Materials and methods

Animals and cell lines. Female, 6- to 8-week-old BALB/c mice were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. BALB/c background H22 hepatocarcinoma cell lines and 435s breast cancer cells were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and cultured according to the guidelines of the CCTCC.

Construction of a short interfering RNA-expressing H22 tumor cell line. A TLR-4 short interfering RNA (shRNA) sequence (TGCATGGATCAGAAACTCAGCAAAG) was constructed into pGenesil-4 vector (Genesil Corp., China), which has four different promoters (mU6, hU6, h7SK and CMV). The recombinant shRNA and control plasmids were transfected into H22 tumor cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Animal experiments and treatment protocols. To analyze the influence of LPS on the growth of H22 hepatocarcinomas, BALB/c mice (n=8 per group) were inoculated with $1x10^5$ H22 cells, which were untransfected or transfected with a TLR-4 shRNA, by subcutaneous injection into the right hind thigh. On the second day after inoculation and every three days thereafter, the mice in the treatment groups received an i.m. injection of 100 μg of LPS. The mice in the control groups received an equal volume of saline. In addition, mice were injected with $2x10^4$ cells, which were untransfected or transfected with TLR-4 shRNA or shRNA TLR-4 combination with COX-2 inhibitor (NS-398; 10 μM) (Biomol Research Laboratories) into the right hind thigh (n=16 per group). On the day of inoculation and every three days thereafter, mice from the treatment groups received LPS (100 μg in 100 μl saline) i.m. When tumors were palpable, the two perpendicular diameters were measured, and the tumor diameter was calculated using the formula $(a \times b^2)/2$, where a is the larger diameter and b is the smaller diameter. In some experiments, the size of the main tumor nodule was measured and the survival of the mice was recorded.

Enzyme-linked immunosorbent assays (ELISA). H22 cells transfected with TLR-4 shRNAs and (or) COX-2 inhibitor (NS-398) were incubated with HSP70 (10 μg/ml) or LPS (10 μg/ml) for 2 h. The culture supernatants were collected, and the amount of TGF-α was measured using a mouse TGF-α ELISA kit (R&D). The experiment was repeated three times.

Analysis of gene expression by RT-PCR and real-time PCR. Total RNAs were isolated using TRIzol reagent (Invitrogen) from cells or muscle tissues from mice according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was used to detect the TLR-4 mRNA as described previously (12): TLR-4, sense 5'-GTGGAAGTTGAACGAATGGA-3'; antisense 5'-TGAAGATGATACCAGCACGAC-3'; and β-actin, sense 5'-ATGGGTCAGAAGGACTCCTATG-3', antisense 5'-ATCTCCTGCTCGAAGTCTAGAG-3'. The results were expressed as the expression level of each gene relative to that of housekeeping gene β-actin.

Quantitative real-time PCR for the IL-6, TGF-β1, TNF-α, VEGF and β-actin genes was performed using procedures previously described (13). The primer sequences were as follows: IL-6, sense 5'-GAGAGGAGACTTCACAGAGGA TAC-3', antisense 5'-GTACTCCAGAAGACCAGAGG-3'; TGF-β1, sense 5'-TGGCTTCTAGTGCTGACGC-3', antisense 5'-TAGTTTGGACAGGATCTGGC-3'; TNF-α, sense 5'-GAC ACCATGAGCACAGAAAG-3', antisense 5'-GAGTAGACAA GGTACAACCC-3'; VEGF, sense 5'-CTTTCTGCTCTCTT GGGTGC-3', antisense 5'-CATGGTGATGTTGCTCTCTG-3'; and β-actin, sense 5'-ATGGGTCAGAAGGACTCCTATG-3', antisense 5'-ATCTCCTGCTCGAAGTCTAGAG-3'. The results were expressed as the expression level of each gene relative to that of housekeeping gene β-actin.

Matrigel invasion assay. The matrigel invasion assay was performed using modified Boyden chambers with a 6.5-mm diameter, porous (8.0 Am) polycarbonate membrane separating the two chambers (Transwell, Corning, Inc., Corning, NY). The transwell filter inserts (Costar) were coated with matrigel. Incubation with LPS (10 μg/ml) was performed after *in vitro* transfection; 435s cells, which were transfected with TLR-4 shRNAs or shRNA TLR-4 combination with COX-2 inhibitor (NS-398; 10 μM), were placed ($2.5x10^5$ cells) in the upper compartment. After 48 h of incubation, 435s cells that had migrated to the lower surface of the membrane were counted, and the percentage of cells that migrated was calculated.

Western blot analysis. After incubation in the presence or absence of LPS after *in vitro* transfection, H22 cells were transfected with TLR-4 shRNAs and/or COX-2 inhibitor (NS-398) or shRNA TLR-4 combination with COX-2 inhibitor (NS-398; 10 μM) and were lysed or homogenized for Western blot analysis, as described (12). Primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA), R&D systems (Minneapolis, CA) and Cell Signaling (Beverly, MA).

Immunohistochemistry. Muscle tissues from the inoculation sites of the treated mice were surgically excised. For indirect immunostaining, fresh tissues were embedded in an optimum cutting temperature solution and cut into 10-μm sections. The sections

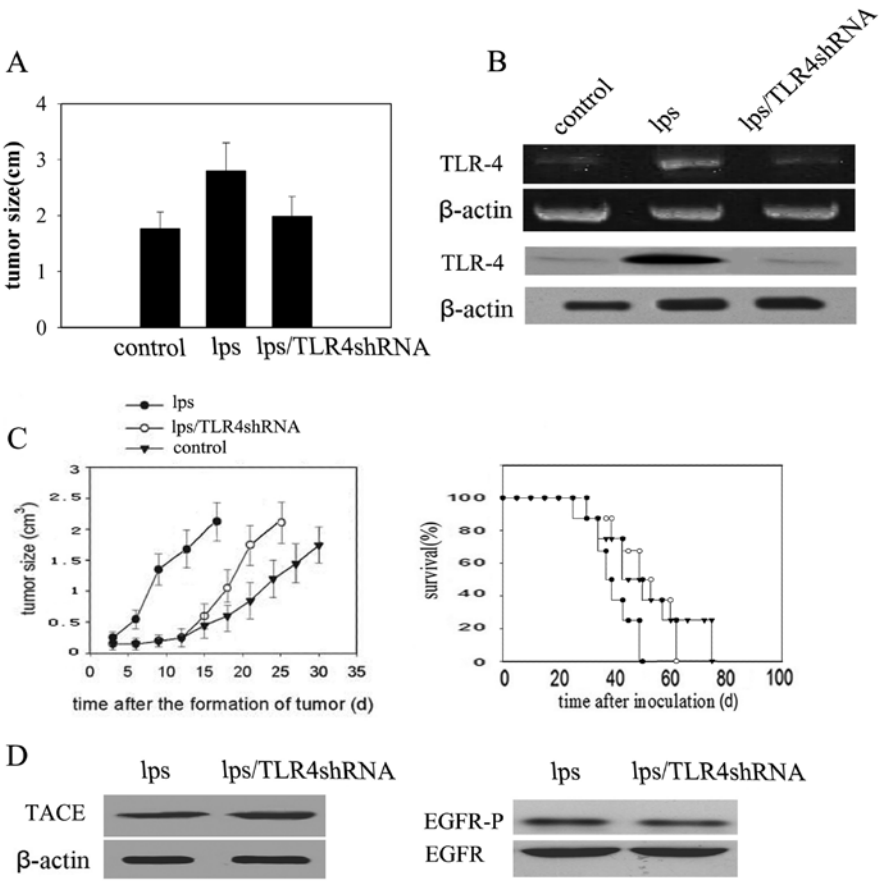

Figure 1. LPS increased the growth of H22 hepatocarcinomas through TLR-4-specific shRNAs. (A) LPS effect on H22 hepatocarcinoma by TLR-4-specific shRNAs. BALB/c mice were inoculated with $1x10^5$ WT or TLR4 shRNAs expressing H22 cells into the right hind thigh muscle and treated with LPS, as described in Materials and methods. The size of each tumor was measured on d16 after inoculation. (B) Twenty-four hours after LPS stimulation, the tissues were isolated from the injection sites of the muscle, and TLR-4 mRNAs and proteins were detected by RT-PCR and Western blot analyses. (C and D) A blockade of TLR4 signaling increased tumor growth and activated TACE after LPS stimulation. Mice (n=16) were inoculated with $2x10^4$ WT or TLR4 shRNA-expressing H22 cells into the right hind thigh muscle and treated with LPS, as described in Materials and methods. $^*P<0.05$, compared to the untransfected group. Animals were monitored every day for survival. The data shown are representative of two experiments. After four weeks, the size of each tumor was measured. Tumor tissues were isolated from the injection sites, and the expression levels of TACE and EGFR proteins were detected by Western blot analysis.

were fixed by acetone and incubated overnight at 4˚C with rat anti-mouse ADAM17 monoclonal antibody diluted at 1/100. Biotinylated rat anti-mouse immunoglobulin G was used as the secondary antibody, followed by streptavidin-conjugated horseradish peroxidase in the third step. The antibodies were purchased from Santa Cruz.

Data analysis. The results are expressed as the mean values ± SD and interpreted by the ANOVA-repeated measures test. Differences were considered to be statistically significant at $P<0.05$.

Results

LPS increases the malignancy of tumor cells in which TLR-4-specific shRNAs inhibited the activation of EGFRs. It has been demonstrated that LPS, a TLR4 ligand, is associated with enhanced tumor growth (13). After inoculating $1x10^5$ H22 cells into the hind thigh muscle and treating mice with lipopolysaccharide, tumor growth was increased significantly after two weeks of treatment with LPS and through the upregulation of TLR4. We also found that transfection of TLR4 shRNAs into H22 cells following LPS stimulation significantly suppressed the increased tumor growth *in vivo* (Fig. 1A and B). However, the effect of inhibition of the TLR4 signal alone is limited and was not enough to eradicate the tumors *in vivo*. To explore the developmental potential of tumor cells by the inhibition of TLR4, we repeated the above experiment by inoculating mice with $2x10^4$ H22 cells and analyzed the efficacy of the inhibition of TLR4. The TLR4-inhibited group treated with LPS stimulation displayed similar trends in tumor growth and had tumors that reached the size of the group that had four weeks of LPS stimulation (Fig. 1C). We also checked the effect of TACE by analyzing the EGFR activation in the tumor tissues. Surprisingly, we found that the tumor tissues expressing TACE and activated EGFR showed no significant change from the control group (Fig. 1D). Together, the results suggest that LPS stimulation in tumor cells might result in a previously unrecognized activation of ADAM17/EGFR, independent of TLR-4.

Proinflammatory cytokine HSP70 stimulation results in the upregulation of TACE and activates EGFR signaling kinase. LPS can induce EGFR activation in lung cancer cells via TNF-α-converting enzyme (TACE)-dependent release of the

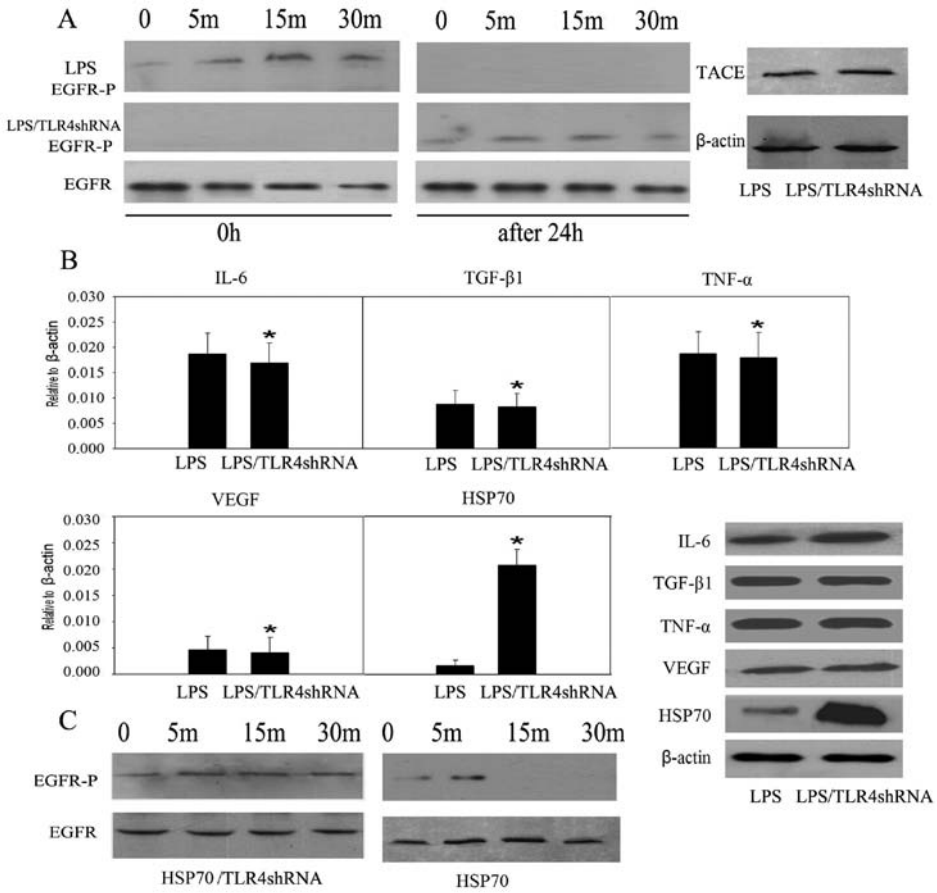

Figure 2. TLR4 signaling was blocked in H22 cells in response to LPS. (A) After H22 cells or TLR-4-inhibited H22 cells were activated with LPS (10 μg/ml) for 0 or 24 h, the effects of TLR-4 inhibition on TACE and activation of EGFR in H22 cells in response to LPS were assayed using Western blot analysis for TACE and EGFR protein levels in H22 cells. (B) Cytokine and growth factor expression was seen in H22 cells after lipopolysaccharide stimulation. H22 cells or TLR-4-inhibited H22 cells were activated with lipopolysaccharide (10 μg/ml) for 24 h to induce the expression of cytokine and growth factor mRNAs and proteins, which were analyzed by real-time PCR and Western blot analysis. *P<0.05, compared to the control group. (C) TLR-4-inhibited H22 cells were activated with HSP70 (10 μg/ml) and analyzed for EGFR activation using Western blot analysis, compared to the H22 cells.

EGFR ligand TGF-α by binding to its putative receptor, TLR-4 (3). However, within 30 min of the LPS stimulation of H22 cells, rapid increases in EGFR phosphorylation and TACE activation were induced. Next we determined how EGFR activation in tumor cells inhibited TLR4. The inhibition of the TLR4 signaling pathway was checked by analyzing the phosphorylation of downstream signaling molecules. The kinetics of the phosphorylation of EGFR and TACE in tumor cells after 24 h of LPS stimulation were very similar to the control group (Fig. 2A); however, within 30 min of LPS stimulation, the EGFR activation was suppressed (Fig. 2A). Interestingly, after knockdown of the TLR4 expression, tumor cell expression levels of IL-6, TGF-β1, TNF-α and VEGF were not significantly different from the control group in response to lipopolysaccharide stimulation (Fig. 2B), but there was a striking induction of HSP70 (Fig. 2B). HSP70 expression is closely correlated with tumorigenesis, malignant development, tumor immunity, resistance to apoptosis and poor clinical prognosis (14-16). Therefore, we speculated that the upregulation of HSP70 in the tumor microenvironment might be responsible for the growth of the tumor. To test this, we examined the effect of exogenously delivered HSP70 on EGFR signaling, and we found that the stimulation of H22 cells by the inhibition of TLR4 induced rapid EGFR phosphorylation (Fig. 2C). From these results, we conclude that HSP70 might be responsible for a bypass feedback loop of EGFR activation in H22 cells through TLR4 inhibition.

A bypass feedback loop of EGFR activation in H22 cells induced TACE-dependent release of HSP70/COX-2. On the basis of the above data, LPS stimulates EGFR phosphorylation (first phase, within 30 min), increased production of HSP70 in H22 cell by TLR-4 specific inhibition, which then induces EGFR activation (second phase, after 24 h) (3,17), and HSP70 can trigger EGFR activation (18) and upregulate the expression of COX-2 (19). The metalloprotease TACE mediates multiple stimuli-induced EGFR activations via the cleavage of the EGFR proligand (e.g., pro-TGF-α) (3,20,21). We wondered whether TACE mediates the COX-2 induced TGF-α release and the second phase of EGFR activation in H22 cells with TLR-4-inhibited shRNAs, which is caused by HSP70 production, Then, we found that stimulating H22 cells by TLR-4-specific inhibition with HSP70 induced rapid EGFR phosphorylation, upregulated COX-2 expression and induced TGF-α release (Fig. 3). Next, we pretreated cells with the selective COX-2 inhibitor (NS-398) to inhibit TGF-α release and suppress HSP70-induced EGFR phosphorylation in H22 cells of TLR-4 inhibition lines, due to the upregulation of COX-2 expression

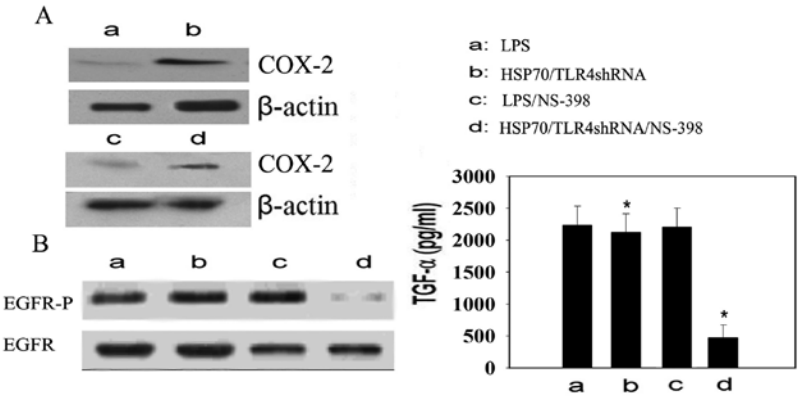

Figure 3. Expression of COX-2 and bypass signaling loop activation of EGFR in H22 cells. (A) H22 cells or TLR-4-inhibited H22 cells were incubated with the COX-2 inhibitor (NS-398; 10 μM) for 30 min and then incubated with LPS (10 μg/ml) or HSP70 (10 μg/ml) for 24 h, followed by Western blot analysis for COX2 protein levels in H22 cells. (B) Effect of inhibition of the TACE-TGF-α cascade on HSP70-induced EGFR activation. Cells were incubated with the COX-2 inhibitor (NS-398; 10 μM) for 30 min and then incubated with LPS (10 μg/ml) or HSP70 (10 μg/ml) for 24 h, followed by Western blot analysis for EGFR activation in H22 cells or TLR-4-inhibited H22 cells (left). Supernatants were collected to measure TGF-α expression using ELISA (right). *P<0.05, compared to the control group.

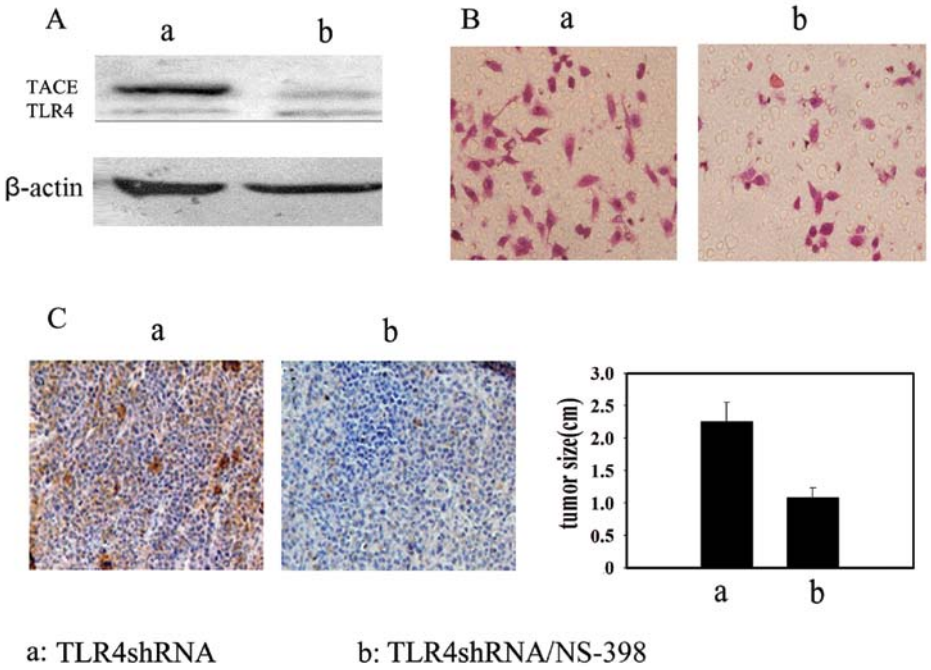

Figure 4. shRNA plasmids containing TLR-4 combination with COX-2 inhibitor reduce the malignant potential of tumor cells in LPS stimulation. (A and B) Following transfection with shRNAs and then incubation with LPS (10 μg/ml) for 24 h, Western blot analysis was used to analyze the TLR-4 and TACE expression levels in H22 cells transfected with shRNAs. In addition to treatment with LPS, as described in Materials and methods, the 435s cells were H&E stained for adhesion to the matrigel-coated plate. (C) Mice (n=8 for the treatment group) were inoculated with $1x10^5$ H22 cells that had been transfected with shRNAs. Immunohistochemistry was used to analyze targeting TACE (left), and the size of each tumor (right) was measured on d16 after inoculation and treated with LPS, as described in Materials and methods. *P<0.05, compared to the singly transfected groups.

(Fig. 3). The LPS-induced TGF-α release and EGFR phosphorylation in H22 cells were not suppressed by the inhibitors but not induced COX-2 expression (Fig. 3). From these results, we concluded that COX-2 inhibitor (NS-398) that prevented the second, but not the first, phase of LPS-induced activation of EGFR via TLR-4 inhibition derived from HSP70/COX-2 production in H22 cells.

COX-2 inhibitor combination with TLR4-specific shRNA inhibition reduces the malignant phenotype of tumor cells following LPS stimulation. The above data show that shRNA TLR-4 inhibited the effects of the first phase of EGFR phosphorylation. Subsequent activation of the HSP70-dependent bypass feedback loop of EGFR phosphorylation in response to LPS and COX-2/TACE activation is a critical step in tumor progression and bypass EGFR activation. This finding suggests that targeting COX-2/TACE signaling inhibition might remedy this effect. To verify this, we used shRNA TLR-4 combination with COX-2 inhibitor. Western blot analysis revaled significantly decreased TLR-4 and TACE expression levels in H22 cells transfected with shRNA TLR-4 combination with COX-2 inhibitor compared to the singly transfected with shRNA TLR-4

group, both groups had LPS stimulation (Fig. 4A). The migration assay showed that in 435s cells transfected with shRNA TLR-4 combination with COX-2 inhibitor, the migration ability of tumor cells *in vitro* (Fig. 4B) was slightly suppressed. In addition, our results showed that transfection of TLR-4 into H22 cells combined with COX-2 inhibitor following LPS stimulation inhibited the mediated activation of TACE and completely abolished the increased tumor growth *in vivo* (Fig. 4C).

Discussion

The major contribution of this study is the demonstration that Hsp70 secreted from cells at initial steps of LPS induction might have the same function by mediating EGFR transactivation. Previously, it has been demonstrated that LPS/TLR-4 stimulates EGFR pathways, which subsequently cause TACE production (1-3), which is strongly associated with malignant tumors (2). Our current studies expand the previous discovery that LPS can induce EGFR phosphorylation (30 min) via the TLR-4-TACE cascade in many cells (3) to include H22 cells. Inhibition of TLR-4 and its downstream signaling cascade cannot effectively suppress the tumor, so complete tumor eradication rarely occurs. Our finding that sustained LPS and knockdown of TIR-4 in H22 cells (by the intramuscular injection of a small number of tumor cells) not only triggered a bypass feedback loop in the second phase of EGFR phosphorylation but also relieved the repression that contributed to the growth of the tumor mass. This might provide a mechanistic link between chronic inflammation and cancer and could lead to potential applications for cancer prevention.

Growing amount of data suggests that a bypass feedback loop with EGFR signaling may initiate a malignant tumor phenotype. We propose that under LPS stimulation conditions the released Hsp70 triggers signaling events using inhibition of TLR-4 which a bypass feedback loop with EGFR in a manner similar to that demonstrated for growth hormone (6). HSP70 is abundantly expressed in malignant tumors of various origins (14-16) and HSP70 promotes tumorigenesis via upregulating the expression of COX-2 and activating the EGFR (19,22). In our study that sustained LPS and knockdown of TIR-4 in H22 cells induced tumor growth was initiated by accumulated HSP70 secretion and induced COX-2 up-regulation that required EGFR activation, which occurs 24 h after stimulation. In H22 cells, LPS stimulation did not induce COX-2 upregulation significantly and only induced the early phase of EGFR activation (30 min). Our studies also found that the COX-2 inhibitor NS-398 inhibited the second, but not the first phase of EGFR phosphorylation.

The EGFR ligand TGF-α is synthesized as a transmembrane precursor molecule that requires proteolytic cleavage by transmembrane metalloproteases known as ADAMs. TACE-mediated shedding of TGF-α is required for EGFR activation (23-25). In the present study, the HSP70/COX2 induced TGF-α release was blocked by the COX-2 inhibitor (NS-398) but not LPS. Together, the data indicated that LPS initiated cross-communication with TACE/TGF-α-EGFR activation is an important signaling cascade of TACE-induced tumor progression. Moreover, we designed the shRNA vector of TIR-4 combination with COX-2 inhibitor. The TACE suppression ratio for protein indicated that singly shRNA vectors of TIR-4 have relatively strong inhibitory effect and may be useful for the prevention of cancer development in tissues with chronic inflammation that are associated with sufficiently blocked bypass feedback loop of EGFR signaling and the cells reverted to the malignant phenotype (26). Future studies will address a variety of potential outcomes for this important cross-communication with EGFR pathway, including carcinogenic effects and effects on cellular differentiation. Thereby, clarification of this signaling cross-communication may provide more clues for understanding the pathophysiology of inflammation and carcinogenesis.

Acknowledgements

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