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Catecholamine-Induced β2-Adrenergic Receptor Activation Mediates Desensitization of Gastric Cancer Cells to Trastuzumab by Upregulating MUC4 Expression

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Trastuzumab is currently used for patients with Her2+ advanced gastric cancer. However, the response rate to trastuzumab among the patients is low. The molecular mechanisms underlying trastuzumab resistance in gastric cancer are unknown. Our in vitro data show that activation of β2-adrenergic receptor (β2-AR) triggered by catecholamine caused “targeting failure” of trastuzumab in gastric cancer cells. The antitumor activities of trastuzumab were significantly impeded by chronic catecholamine stimulation in gastric cancer cells and in the mice bearing human gastric xenografts. Mechanistically, catecholamine induced upregulation of the MUC4 expression at both transcription and protein levels via activating STAT3 and ERK. The effects of catecholamine could be effectively blocked by β2-AR antagonist ICI-118,551, indicating that β2-AR-mediated signaling pathway plays a key role in upregulation of MUC4, which was previously demonstrated to interfere with the recognition and physical binding of trastuzumab to Her2 molecules. Moreover, a significant elevation of the MUC4 level was observed in the xenograft tissues in nude mice chronically treated with isoproterenol. Knockdown of MUC4 restored the binding activities of trastuzumab to Her2-overexpressing gastric cancer cells. In addition, coexpression of β2-AR and MUC4 were observed in gastric cancer tissues. Our data indicated a novel trastuzumab resistance mechanism, by which catecholamine-induced β2-AR activation mediates desensitization of gastric cancer cells to trastuzumab through upregulating the MUC4 expression. The Journal of Immunology, 2013, 190: 5600–5608.

Gastric cancer is the second most common cancer worldwide, with an estimated >900,000 new cases per year (1). Although the incidence and mortality of gastric cancer have decreased markedly in most areas of the world over the past several decades, control of gastric cancer at the advanced stage remains difficult (2). In 2010, the Food and Drug Administration and European Medicines Agency approved therapeutic Ab (Herceptin) use for patients with Her2-overexpressing metastatic gastric cancer. Potentially, ∼25% of the patients may benefit from trastuzumab therapy, because these patients are Her2+.

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Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; β2-AR, β2-adrenergic receptor; ATEN, atenolol; ISO, isoproterenol; PCNA, proliferating cell nuclear Ag; PKA, protein kinase A; pRL-TK, thymidine kinase promoter- Renilla luciferase reporter plasmid; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00 (3, 4). However, the response rate to trastuzumab among Her2+ advanced gastric cancer patients is only 12.8% (5). It was reported that the trastuzumab-mediated Ab-dependent cellular cytotoxicity (ADCC) was significantly impaired and the decreased effect of trastuzumab was attributed to NK cell dysfunction in gastric cancer patients (6). The molecular mechanisms underlying intrinsic or acquired trastuzumab resistance in gastric cancer are unknown.

Several possibilities for trastuzumab resistance in breast cancer have been proposed (7–10), including persistent activation of the PI3K pathway (11–13), cross talk of heterologous receptor signaling pathways (14), and cleavage of Her2 extracellular domain (15). It is unclear whether similar mechanisms of trastuzumab resistance act in gastric cancer. Numerous clinical studies demonstrated that chronic psychological stress profoundly affected the malignant progression of cancer and therapeutic outcome (16–18). The activation of β2-adrenergic receptor (β2-AR) signaling pathway induced by catecholamine is considered as a key event occurring under stress (19). In our previous study, we show that β2-AR was overly expressed in some gastric cancer tissues, particularly in metastatic tissues, suggesting a critical role of the activation of β2-AR in the invasion and metastasis of gastric cancer (20). We also demonstrated that Her2 and β2-AR comprise a positive feedback loop in breast cancer (21). Cross talk between Her2 and β2-AR may activate a complex intracellular signaling network, which may contribute to the drug resistance and failure of targeted therapeutics.

In this study, we investigated the effects of the β2-AR activation on the antiproliferation activities of trastuzumab in gastric cancer and revealed the potential mechanisms of trastuzumab resistance in gastric cancer. Our data indicated that β2-AR mediates desensitization of gastric cancer cells to trastuzumab by upregulating the MUC4 expression.

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Materials and Methods

Cell culture and treatment

Human gastric cancer cell lines NCI-N87, MGC-803, and HGC-27 were obtained from the American Type Culture Collection. Human gastric cancer cell line BGC-823 was a gift from Prof. Zhihua Yang (Chinese Peking Union Medical College, Chinese Academy of Medical Science). For treatment with β2-AR agonists or antagonists, the cells were incubated overnight in a serum-free medium and then treated with 2 μM epinephrine (Sigma), 0.1–10 μM isoproterenol (ISO; Sigma), 5 μM propranolol (Sigma), 1 μM ICI-118,551 (Sigma), or 5 μM atenolol (ATEN) for the indicated time points.

Construction

The promoter region (nucleotides –2986 to –1 relative to the ATG initiation codon) of human MUC4 gene was PCR amplified from the genomic DNA of NCI-N87 cells using the primers (sense: 5′-CGGGGTACCTTA-CACCTGACTCTGACCTCTG-3′ and antisense: 5′-C CCCAGGTCTT-GCGGCAAAGTCCCTCGGCT-3′) with Power Pfu DNA polymerase (BioTeKe) and ligated to the immediate upstream of a firefly luciferase gene in pGL3 basic vector (Promega) designated pGL3/MUC4.

Western blot

The following Abs were used: the rabbit polyclonal Abs against β2-AR (Abcam) and Her2 (Cell Signaling), and the mouse mAbs against MUC4 (BioTeke) and ligated to the immediate upstream of a firefly luciferase gene in pGL3 basic vector (Promega) designated pGL3/MUC4.

Conventional and quantitative RT-PCR

NCI-N87 and HGC-27 cells were treated with 10 μM ISO for 0, 1, 6, and 12 h. The total RNA was isolated from NCI-N87 and HGC-27 cells using TRIzol reagent (Invitrogen). cDNAs were synthesized by reverse transcription kit (BioTeke) in accordance with the manufacturer’s instructions. Conventional RT-PCR was used to detect the expression of MUC4 mRNA induced by ISO with the specific primers (sense: 5′-GCCAAGCTTA-CAGTGTGACTCA-3′ and antisense: 5′-ATGGTGCCGGTTGTAATTGGTG-3′). Amplification of β-actin with the specific primers (sense: 5′-GTGGGGCCGCCCCACCCAGCACA-3′ and antisense: 5′-CTCTCCTTATG-TACGCAGGATTTC-3′) was used as the control. The effect of ISO on MUC4 gene expression was validated by real-time quantitative RT-PCR using SYBR Green Supermix (TIANGEN Biotech) on Real-Time PCR Detection System (Agilent Technologies) as recommended by the manufacturer. The results were analyzed using the comparative threshold cycle method with β-actin as an internal control. The experiment was carried out at least twice.

Transfection and luciferase assays

NCI-N87 cells were cotransfected with pGL3/MUC4 and the thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After transfection for 48 h, the cells were starved in serum-free medium for an additional 24 h and then treated with the STAT3 small interfering RNA (siRNA), MEK inhibitor PD98059, protein kinase inhibitor H89, β-AR inhibitor propranolol, β2-AR inhibitor ICI-118,551, or β1-AR inhibitor ATEN followed by stimulation with 10 μM ISO for the indicated time points. The luciferase activities were measured using a dual luciferase assay kit (Promega) according to the manufacturer’s instructions. The experiment was performed in duplicate.

Flow cytometric analysis

A total of 1 × 10^6 cells were collected for each sample. The cells were gently harvested (22) and incubated with trastuzumab (5 μg/ml) at 4°C for 30 min. After washing with PBS for three times, the cells were incubated with FITC-conjugated anti-human IgG at 4°C for 30 min. After washing, cells were resuspended in PBS containing 1% formaldehyde. The fluorescence intensity was determined by flow cytometry (Becton-Dickinson). The experiment was performed in duplicate.

Immunohistochemistry

Paraffin-embedded tissue sections were dewaxed and gradually hydrated. Endogenous peroxidase activities were quenched using 3% hydrogen peroxide. After Ag retrieval, the sections were incubated with the rabbit mAb against Her2 (Cell Signaling), and rabbit polyclonal Abs against β2-AR (Abcam) and CD31 (Abcam). After washing with PBS, the sections were incubated with HRP-conjugated goat anti-rabbit Ab. The color was developed by incubation with 3,3′-diaminobenzidine solution. Photomicrographs were taken with an Olympus microscope BX53. Omission of the primary Ab and substitution by non-specific rabbit IgG at the same concentration were used as negative controls.

ADCC

An ADCC assay was performed by lactate dehydrogenase release assay using human PBMCs as the effector cells and NCI-N87 cells as the target cells at an E:T ratio of 20:1, as described previously (23). The target cells were treated with 10 μM ISO at 37°C for 9 h followed by washing with PBS. Then 1 or 2 μg/ml trastuzumab and the effector cells were added. Alternatively, the target and effector cells were incubated with trastuzumab and ISO simultaneously at 37°C for 4 h. The percentage of cytolysis was calculated as [(Sample release – spontaneous release effector – spontaneous release target)/maximum release target] × 100%. The experiments were repeated three times.

Proliferation assay

NCI-N87 and HGC-27 cells were cultured in 96-well plates with an initial cell density of 4 × 10^3/well in DMEM containing 0 or 2.5 μM ISO and 0 or 5 μg/ml trastuzumab for indicated time points. In vitro proliferation activities were measured by CCK8 assays following the manufacturer’s instructions. The experiment was performed in duplicate.

In vivo tumor model

Five- to 6-wk-old female athymic BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology. The mice were divided into two groups randomly and each group contained 10 mice. The mice received PBS or ISO (10 mg/kg; Sigma) (24) by daily i.p. injections commencing 2 d before inoculation of tumor cells. A total of 0.1 ml NCI-N87 cell suspension (10^7 cells/ml) was injected s.c. in the right upper flank of the mice. Trastuzumab treatment was started 10 d after implantation of NCI-N87 cells. The mice treated with PBS were further divided into two groups (five mice per group) randomly and trastuzumab (0.5 mg/mouse; Genentech) (14) or IgG (0.5 mg/mouse) was given to mice i.p. twice a week for 4 wk. The mice treated with ISO were further grouped (five mice per group) randomly and cotreated with trastuzumab (0.5 mg/mouse, twice a week) and ISO (10 mg/kg, daily) or IgG (0.5 mg/mouse, twice a week) and ISO (10 mg/kg, daily) i.p. for 4 wk. Forty days after tumor implantation, mice were sacrificed. Primary tumors were dissected, weighted, photographed, and fixed in formalin. The expression of β2-AR, Her2, and CD31 in primary tumors was assayed by immunohistochemistry, and the expression of MUC4 and phosphorylation of STAT3 was determined by Western blot.

Statistical analysis

Data were expressed as mean ± SD. Paired data were evaluated by Student t test. For comparisons between multiple groups, two-way ANOVA tests were used. A p value <0.05 was considered statistically significant.

Results

β2-AR is overexpressed in human gastric cancer tissues and is associated with cellular proliferation

In our previous study, we showed that catecholamine induces MMP-7 expression in gastric cancer, implicating that β2-AR-mediated signaling pathway is involved in invasion and metastasis of gastric cancer (20). In this study, we examined by immunohistochemistry the expression of β2-AR in 15 human gastric cancer tissues. β2-AR is highly expressed (+++ or ++) in nine gastric cancer tissues. Fig. 1A and 1B show the representative images. Stronger staining was observed mainly at the invasive front of the tumor tissues (Fig. 1A). Notably, many β2-AR–overexpressing cells appeared to be mitotically active (Fig. 1B). In contrast, the expression of β2-AR was low or undetectable in the relatively normal gastric gland cells (Fig. 1C). To determine the correlation of the β2-AR expression with tumor cell proliferation, we analyzed the expression of β2-AR and proliferating cell nuclear Ag (PCNA) on serial sections. Interestingly, the tumor cells overexpressing β2-AR frequently exhibited positive staining of PCNA, whereas PCNA was undetectable in the cells lacking β2-AR expression (Fig. 1C), implicating that vigorous proliferation takes place especially in the β2-AR–overexpressing...
gastrointestinal cancer cells, and that β2-AR–mediated signaling pathway participates in the regulation of cellular proliferation. Another interesting finding was that the β2-AR expression was associated with poor differentiation, as β2-AR was distinctively expressed in numerous intestinal-type crypts and goblet cells, a hallmark of intestinal metaplasia, and poorly differentiated tumor area. In addition, the extensive infiltration of inflammatory cells, including neutrophils and lymphocytes, was pronounced around the β2-AR–overexpressing region, concomitant with an increase in microvascular permeability. Scale bars, 100 μm.

Our previous study demonstrated that β2-AR and Her2 were coexpressed in human breast cancer tissues (21) and suggested that the interaction of the heterogeneous receptors may influence breast cancer progression. Her2 overexpression is increasingly recognized as a frequent molecular abnormality in gastric cancer by gene amplification (4). We first screened the expression of Her2 and β2-AR in a panel of human gastric cancer cell lines NCI-N87, BCG-823, MGC-803, and HGC-27. Fig. 2A shows that the expression of β2-AR was detectable in all cell lines tested. Coexpression of β2-AR and Her2 was seen in NCI-N87 and HGC-27 cells. Thus, NCI-N87 and HGC-27 cells were selected as the cellular models for further investigation. Because aberrant expression of MUC4, a membrane-associated, highly glycosylated mucin (31), is observed frequently in gastric cancers (32, 33), we also analyzed the expression of MUC4 in these cells. Surprisingly, when Her2 and β2-AR double-expressing NCI-N87 cells were treated with β-AR agonists (2 μM epinephrine and 10 μM ISO), the level of MUC4 was remarkably increased in a time-dependent manner (Fig. 2B). Similar data were obtained in HGC-27 cells (Fig. 2C). To verify the upregulation of MUC4 by catecholamine, we treated NCI-N87 and HGC-27 cells with different concentrations of ISO (0, 0.1, 0.3, 0.6, 1.2, 2.5, 5, or 10 μM). Western blot analysis shows that the MUC4 level was stably elevated in a dose-dependent manner (Fig. 2D). Upregulation of MUC4 expression by ISO was also validated by immunofluorescent staining (Supplemental Fig. 1). Furthermore, the effects of β2-AR agonist...
on the MUC4 expression could be effectively blocked by β2-AR antagonist ICI-118,551 (Fig. 2E), indicating that the MUC4 expression is influenced by β2-AR–mediated signaling pathway.

**Catecholamine upregulates MUC4 expression at the transcription level**

To determine whether the expression of MUC4 is upregulated at the transcription level, we examined MUC4 mRNA expression after ISO stimulation by conventional and real-time RT-PCR. As shown in Fig. 3A, MUC4 mRNA expression was dramatically increased by 2-fold at 1 h after ISO stimulation, reaching a maximum at 6 h (~5- to 6-fold increase). We then constructed the luciferase reporter plasmid containing the promoter sequences of MUC4 (pGL3/MUC4) and transfected NCI-N87 cells with this plasmid. Luciferase assays show that MUC4 promoter activities started to increase at 1 h after ISO induction, peaked at 3 h, and then decreased after 6 h of induction (Fig. 3B). The effects of ISO on MUC4 transcription could be strongly inhibited by the β-AR inhibitor propranolol and β2-AR inhibitor ICI-118,551, but not by the β1-AR antagonist ATEN (Fig. 3C). Similarly, ISO-induced MUC4 promoter activities were also specifically suppressed by propranolol and ICI-118,551 (Fig. 3D). The data demonstrate that catecholamine stimulates the transcription of MUC4 gene via activation of β2-AR.

**FIGURE 3.** Catecholamine upregulates MUC4 in gastric cancer cells at the transcription level. (A) NCI-N87 and HGC-27 cells were treated with 10 μM ISO. The expression of MUC4 mRNA was detected by conventional and real-time RT-PCR at the indicated time points. (B) NCI-N87 cells were cotransfected with the plasmids pGL3/MUC4 and pRL-TK. Luciferase activities were analyzed at the indicated time points. (C) NCI-N87 and HGC-27 cells were incubated overnight in a serum-free medium and then treated with 2.5 μM ISO in the presence or absence of 5 μM propranolol or 1 μM ICI-118,551 or 5 μM ATEN. MUC4 mRNA expression was analyzed by real-time RT-PCR. (D) NCI-N87 cells were starved and then treated with 2.5 μM ISO in the presence or absence of 5 μM propranolol or 1 μM ICI-118,551 or 5 μM ATEN. Luciferase activities were analyzed. *p < 0.05, **p < 0.01.
**β2-AR activation mediates trastuzumab resistance**

β2-AR is an important member of seven-transmembrane G protein–coupled receptors (19). The previous studies showed that norepinephrine or epinephrine enhanced the phosphorylation of STAT3 and induced its nuclear translocation through activating β2-AR. It was reported that STAT3 promoted IL-6–induced MUC4 expression by directly binding to the MUC4 promoter (33, 34). Our previous study demonstrated that catecholamine induced the expression of MMP-7 by activating STAT3 and AP-1 in gastric cancer cells (20). To verify the role of STAT3 activation in catecholamine-induced upregulation of MUC4 expression, we assessed the phosphorylation of STAT3 in NCI-N87 and HGC-27 cells. Fig. 4A shows that ISO stimulation induced persistent phosphorylation of STAT3 and ERK. Knockdown of STAT3 by the specific siRNA fully abrogated MUC4 upregulation in response to ISO stimulation (Fig. 4B). Similarly, the treatment with JAK2 tyrosine kinase inhibitor tyrphostin AG490 also efficiently abolished ISO-induced MUC4 expression in NCI-N87 and HGC-27 cells (Fig. 4C), indicating that catecholamine upregulated the MUC4 expression through activating STAT3. In addition, the ERK signaling pathway was also involved, because the level of ERK phosphorylation was significantly enhanced by ISO stimulation and MEK1 inhibitor PD98059 blocked ISO-induced MUC4 expression. It has been known that β2-AR can initiate the activation of STAT3 and ERK through cAMP/protein kinase A (PKA) signaling (19). When we treated the cells with protein kinase inhibitor H89, ISO-induced MUC4 expression was conspicuously reduced. Earlier data indicate that MUC4 expression is repressed. Fig. 4D demonstrates that the activation of the MUC4 promoter by ISO was significantly impaired by the STAT3 siRNA, PD98059 or H89. Earlier data indicate that MUC4 expression is upregulated by β2-AR–mediated activation of STAT3 and ERK via PKA signaling pathway upon agonist stimulation in gastric cancer cells.

**Induction of MUC4 by ISO inhibits the binding of trastuzumab to Her2-overexpressing gastric cancer cells**

A recent study indicated that MUC4 is a modulator of Her2 signaling and a determinant of therapeutic outcome of trastuzumab. It has been reported that the local density of MUC4 is negatively correlated with trastuzumab binding (22, 35). MUC4 is composed of an O-glycosylated mucin subunit and an N-glycosylated transmembrane subunit. The latter contains three epidermal growth factor–like domains, which interact with Her2. The complex formation of MUC4/Her2 may prevent the recognition and physical binding of trastuzumab to Her2 (31, 35). As catecholamine stimulation induced the upregulation of MUC4, we speculated that the effect may interfere with the association of trastuzumab with Her2. We transfected NCI-N87 cells with control or MUC4 siRNA and analyzed the binding activities of trastuzumab by flow cytometry. As demonstrated in Fig. 5A, the binding activities of trastuzumab to the cells were significantly reduced after ISO treatment. Either transfection of MUC4-specific siRNA or pretreatment with H89 effectively inhibited ISO-induced MUC4 expression (Fig. 5B) and restored trastuzumab binding activities to NCI-N87 cells (Fig. 5A), suggesting that the upregulation of MUC4 expression interfered with the interaction of trastuzumab with Her2 molecules, leading to “targeting failure” of trastuzumab in gastric cancer cells. Similarly, ISO stimulation also induced the expression of MUC4 in human ovary cancer cell line SKOV3 (Fig. 5C, left panel) and diminished the binding activities of trastuzumab to SKOV3 cells (Fig. 5C, right panel) as determined by flow cytometric analysis. Notably, ISO induction caused a simultaneous upregulation of both MUC4 and Her2 (Fig. 5C, left panel), indicating that the decrease of binding activities of trastuzumab occurred in the presence of high level of Her2. The earlier data confirm that β2-AR–mediated signaling modules MUC4 expression and blocks trastuzumab targeting.

**ISO induces trastuzumab resistance**

Our previous study showed that catecholamine stimulated the growth of human breast cancer cells (21). However, we noticed that ISO seemed to slightly inhibit the proliferation of NCI-N87 and HGC-27 cells (Fig. 6A). Other in vitro studies also demonstrated that the regulation of cell proliferation by catecholamine was cell-type dependent. As shown in Fig. 6A, ISO significantly antagonized the antiproliferation activities of trastuzumab in NCI-N87 and HGC-27 cells. To determine whether catecholamine inhibits trastuzumab-triggered ADCC, we treated NCI-N87 cells with ISO. As shown in Supplemental Fig. 2A, the capacity of trastuzumab to induce ADCC against ISO-treated NCI-N87 cells was not evidently affected. However, trastuzumab-induced ADCC was greatly inhibited when the effector and target cells were exposed to trastuzumab and ISO simultaneously. A recent study revealed that trastuzumab was able to initiate ADCC response in the cells that express low level of Her2 (36). It was reported that the activities of NK cells and ADCC could be inhibited by β-adrenergic stimulation (29, 37). These data suggest that catecholamine stimulation antagonizes the inhibitory effects of trastuzumab on the proliferation of gastric cancer cells mainly by impairing the target.

**FIGURE 4.** Catecholamine upregulates MUC4 by activating STAT3 and ERK. (A) NCI-N87 and HGC-27 cells were treated with 10 μM ISO. The phosphorylation of STAT3 and ERK was analyzed by Western blot at the indicated time points. (B) NCI-N87 cells were transfected with the STAT3 siRNA and then starved for 24 h followed by stimulation with 10 μM ISO. The expression of MUC4 and STAT3 was examined by Western blot. (C) NCI-N87 and HGC-27 cells were starved and then treated with 50 μM AG490 or 25 μM PD98059 or 10 μM H89 for 1 h followed by stimulation with 10 μM ISO. The expression of MUC4 and phosphorylation of ERK and STAT3 were analyzed by Western blot. (D) NCI-N87 cells were cotransfected with pGL3/MUC4, pRL-TK, and STAT3 siRNA. After 48 h, the transfected cells were starved overnight and treated with 10 μM H89 or 25 μM PD98059 for 1 h followed by stimulation with 10 μM ISO. Luciferase activities were analyzed after ISO treatment for 3 h. **p < 0.01."
binding and reduces trastuzumab-induced ADCC activity by repressing the activities of effector cells.

To investigate whether catecholamine affects the efficacy of trastuzumab in vivo, we treated the mice bearing NCI-N87 xenografts with trastuzumab (0.5 mg/mouse) twice a week, along with ISO injection (10 mg/kg) daily. Fig. 6B shows that trastuzumab efficiently inhibited the tumor cell growth in mice, whereas ISO stimulation markedly impeded the antiproliferative efficacy of trastuzumab. We examined the expression of Her2 and β2-AR in the xenograft tissues by immunohistochemistry. The coexpression of both receptors was clearly seen (Fig. 6C). We also assessed the expression of MUC4 by Western blot. Surprisingly, the level of MUC4 was importantly increased in the xenograft tissues from mice treated with ISO, trastuzumab, or both (Fig. 6D). Nevertheless, stronger expression of MUC4 was detected in ISO-treated groups than in trastuzumab-treated groups. These data demonstrate that chronic ISO stimulation antagonizes the antitumor activities of trastuzumab in vivo.

To explore the possible mechanism underlying the induction of MUC4 by trastuzumab, we examined the levels of phosphorylated STAT3 in the xenograft tissues. As shown in Supplemental Fig. 3A, phosphorylation of STAT3 was remarkably enhanced in the trastuzumab-treated group. We established the trastuzumab-resistant NCI-N87 cells by culturing the cells in sublethal level of trastuzumab over half a year. Surprisingly, STAT3 phosphorylation was dramatically increased and MUC4 expression also upregulated in these cells, compared with the parental cells (Supplemental Fig. 3B). STAT3 plays a key role in MUC4 expression. We hypothesize that activation of STAT3 and upregulation of MUC4 are critical in the development of acquired trastuzumab resistance in gastric cancer cells.

The recent studies indicate that catecholamine-mediated signaling pathways promote tumor angiogenesis by upregulating the synthesis of proangiogenic factors like VEGF. We performed immunohistochemical staining for CD31 to evaluate angiogenesis in NCI-N87 cell xenograft tissues. We observed that the tumor microvessel density was greatly increased in the ISO-treated group compared with the control group (Supplemental Fig. 4; p < 0.01). Antiangiogenesis is one of important antitumor mechanisms of trastuzumab by inhibiting Her-2–mediated VEGF expression (9). The data also support that catecholamine antagonizes antitumor activities of trastuzumab.

β2-AR and MUC4 are coexpressed in human gastric cancer tissues

To further reveal the relationship between β2-AR and MUC4, we examined the expression of β2-AR and MUC4 in human gastric cancer tissues. Coexpression of β2-AR and MUC4 was readily observed in gastric cancer tissues (Fig. 7A). Interestingly, the expression of β2-AR and MUC4 was preferentially distributed in the cells in the proliferative neck region and gland base. Moreover, the aberrant expression of β2-AR and MUC4 predominantly occurred in poorly differentiated area with intestinal metaplasia (Fig. 7B), supporting the hypothesis that the activation of β2-AR associates with the overexpression of MUC4, trastuzumab resistance, and malignant progression of gastric cancer.

Taken together, our study predicts that the activation of β2-AR by catecholamine upregulates the expression of MUC4 at the transcription level via activating STAT3 and ERK through PKA signaling pathway, leading to Her2 epitope masking by overexpressed MUC4 and consequent trastuzumab resistance (Fig. 7C).

Discussion

The combination of trastuzumab with chemotherapy is now standard for patients with Her-2+ advanced gastric cancer. The use of trastuzumab in the metastatic setting has resulted in a clinically and statistically significant benefit (4). However, the uncertainties on the exact molecular mechanisms of trastuzumab actions and its primary or acquired resistance in both breast and gastric cancer remain unresolved (5). Investigations into the mechanisms of trastuzumab resistance must now be expedited to maximize the benefit from this agent.

Gastric cancer is a multifactorial disease (2). Several epidemiological studies suggested that psychological stress factors may accelerate the progression of gastric cancer (38, 39). The association of psychological stress with gastrointestinal symptoms has long been noticed (40). Emotional distress in cancer patients can cause complex physiological and neuroendocrine changes, leading to the activation of the hypothalamic-pituitary-adrenal axis and increased release of stress-related hormones, such as catecholamine (17, 18). Stimulation of catecholamine directly influences biobehaviors of tumor cells, mainly through the β2-AR–mediated signaling pathway (19). However, there is a lack of knowledge...
about the functional significance of β2-AR activation in gastric cancers. The findings in our previous study demonstrate that catecholamine upregulates MMP-7 expression by activating AP-1 and STAT3 in gastric cancer cells. The critical role of β2-AR activation in invasion and metastasis of gastric cancer was implicated (20). In this study, we show that β2-AR was overexpressed in human gastric cancer tissues, and its expression correlated with the proliferation and differentiation in gastric cancer. Tumor cells overexpressing β2-AR may be more sensitive to the stimulation of catecholamine. We noticed that β2-AR and MUC4 were coexpressed in gastric cancer tissues. Importantly, β2-AR and MUC4 were predominantly colocalized in actively proliferative neck regions, where stem cells for the gastric mucosa and proliferating gastric mucosal cells are mainly present. Moreover, overexpression of β2-AR and MUC4 appeared to associate with poor differentiation. Catecholamine stimulation upregulated the level of MUC4 in Her2-overexpressing gastric cancer cells and β2-AR antagonist can effectively block the effect, indicating that β2-AR-mediated signaling pathway plays a key role in upregulating MUC4 in response to catecholamine stimulation.

MUC4 is a multifunctional transmembrane glycoprotein. Aberrant expression of MUC4 has been observed in several primary epithelial malignancies, including gastric cancer (41). Several recent studies have provided strong evidence on the intimate link between MUC4 and Her2. For instance, MUC4 upregulated Her2 level and potentiated the Her2 signaling. The transmembrane subunit of MUC4 was suggested to induce Her2 phosphorylation by interacting directly with Her2 (42–44). The activation of Her2 by MUC4 was observed in poorly differentiated

**FIGURE 6.** ISO induces trastuzumab resistance in vitro and in mice bearing gastric cancer xenografts. (A) NCI-N87 and HGC-27 cells were treated with 2.5 μM ISO and 5 μg/ml trastuzumab. In vitro proliferation activities were measured by CCK8 assays. (B) Five- to 6-wk-old female athymic BALB/c nude mice received PBS or ISO (10 mg/kg) by daily i.p. injections commencing 2 d before inoculation of tumor cells. A total of 0.1 ml NCI-N87 cell suspension (10⁶ cells/ml) was injected s.c. in the right upper flank of the mice. Trastuzumab treatment was started 10 d after implantation of NCI-N87 cells. The mice treated with PBS were further divided into two groups, and trastuzumab (0.5 mg/mouse) or IgG (0.5 mg/mouse) was given to mice i.p. twice a week for 4 wk. The mice treated with ISO were further grouped and cotreated with trastuzumab (0.5 mg/mouse, twice a week) plus ISO (10 mg/kg, daily) or IgG (0.5 mg/mouse, twice a week) plus ISO (10 mg/kg, daily) i.p. for 4 wk. Forty days after tumor implantation, mice were sacrificed. (B) Primary tumors were dissected, weighted, and photographed. (C) The expression of β2-AR and Her2 in primary tumors was assayed by immunohistochemistry. (D) The expression of MUC4 in primary tumors was determined by Western blot. Scale bar, 200 μm. *p < 0.05, **p < 0.01. Trast, Trastuzumab.

**FIGURE 7.** β2-AR and MUC4 are coexpressed in human gastric cancer tissue. (A) The expression of β2-AR and MUC4 in human gastric cancer tissues was examined by immunohistochemistry with the Abs against MUC4 and β2-AR. Middle and right panels are the magnifications of the square regions on the left. Scale bars, 1000 μm (left), 200 μm (middle), 100 μm (right). (B) The aberrant expression of β2-AR and MUC4 was predominantly observed in poorly differentiated area with intestinal metaplasia. Right panels are the magnifications of the square regions in the left panels. Scale bars, 200 μm (left), 100 μm (right). (C) Schematic presentation of the molecular mechanism of trastuzumab resistance in human gastric cancer: the activation of β2-AR by catecholamine upregulates the expression of MUC4 at transcriptional level via activating STAT3 and ERK through PKA signaling pathway, leading to Her2 epitope masking by overexpressed MUC4 and consequent trastuzumab resistance.
gastrointestinal cancer (45). Nonetheless, how MUC4 is upregulated in gastrointestinal cells is not fully understood. It has been shown that proinflammatory cytokine IL-6 induces the MUC4 expression through the gp130/STAT3 pathway in gastric cancer cells. Direct binding of STAT3 to the cis-element in the MUC4 promoter has also been confirmed (34). Our study indicated that upregulation of MUC4 in response to catecholamine stimulation was at the transcription level via activating STAT3 and ERK through PKA signaling pathway.

Several studies have established the association of MUC4 with the resistance to trastuzumab in breast cancer. Ectopic expression of rat MUC4 reduced binding of anti-Her2 Abs to Her2-expressing breast cancer cells (46). It was found that the expression of MUC4 was greatly increased in trastuzumab-resistant JIMT-1 cells, and its level was inversely correlated with the trastuzumab binding capacity. It was suggested that steric hindrance from complex formation of MUC4 and Her2 impaired trastuzumab binding to Her2 (22). This study provides new evidence that catecholamine induces the MUC4 upregulation and trastuzumab resistance in gastrointestinal cancer cells both in vitro and in vivo. The binding of trastuzumab to Her2-expressing gastric cancer cells was remarkably decreased after catecholamine stimulation, which upregulated the Her2 level at the same time, indicating that targeting of Her2 by trastuzumab was critically hindered by overexpressed MUC4 molecules. The expression of MUC4 was markedly increased in xenograft tissues, and the antitumor activities of trastuzumab were significantly impeded in mice treated with ISO. Colocalization of β2-AR/Her2 and β2-AR/MUC4 provide substantial molecular basis for the interaction of heterogenous transmembrane molecules and cross talk among multiple intracellular signaling pathways, linking the activation of β2-AR, enhanced Her2 signaling, and MUC4 overexpression to decreased sensitivities of gastric cancer cells to trastuzumab.

Despite optimization of surgery, radiotherapy, and chemotherapy, survival of advanced gastric cancer is poor. Trastuzumab is often used to treat patients with breast cancer, it is predictable that the occurrence of trastuzumab resistance in gastric cancer (2, 4). Based on the findings in trastuzumab treatment of the JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line.

MUC4 binds to the HER2 receptor with a high specificity and affinity and competitively inhibits trastuzumab binding to HER2, which is critical for the tumor inhibitory effect of trastuzumab (28). We found that both the formation of MUC4 and Her2 impaired trastuzumab binding to gastric cancer cells both in vitro and in vivo. The binding of trastuzumab to Her2-expressing gastric cancer cells was remarkably decreased after catecholamine stimulation, which upregulated the Her2 level at the same time, indicating that targeting of Her2 by trastuzumab was critically hindered by overexpressed MUC4 molecules. The expression of MUC4 was markedly increased in xenograft tissues, and the antitumor activities of trastuzumab were significantly impeded in mice treated with ISO. Colocalization of β2-AR/Her2 and β2-AR/MUC4 provide substantial molecular basis for the interaction of heterogenous transmembrane molecules and cross talk among multiple intracellular signaling pathways, linking the activation of β2-AR, enhanced Her2 signaling, and MUC4 overexpression to decreased sensitivities of gastric cancer cells to trastuzumab.

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