Growth Effect of Neutrophil Elastase on Breast Cancer: Favorable Action of Sivelestat and Application to Anti-HER2 Therapy

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Abstract. *Aim: To investigate the relation between neutrophil elastase (NE) and proliferation of breast cancer cells and whether the NE inhibitor sivelestat could both contribute and be applied to therapy for anti-epithelial growth factor receptor 2 (HER2)-positive breast cancers. Materials and Methods: The proliferation or inhibition of breast cancer cell line SKBR-3 by each agent was evaluated by methylthiazole tetrazolium (MTT) assay. Signal transduction and expression of signaling molecules were evaluated by Western blot analysis. Results: The auto tumor progression mechanism initiated by NE through tumor growth factor-α (TGF-α) was present in breast cancer cells, and this mechanism was intensively suppressed by sivelestat. The effect of trastuzumab was suppressed, and trastuzumab-induced HER2 down-regulation was impaired by TGF-α. TGF-α not only promoted cell proliferation as a ligand but also enhanced resistance to trastuzumab by impairing HER2 down-regulation. Furthermore, combined use of trastuzumab and sivelestat suppressed cell proliferation more intensively than either drug alone and did not provoke impairment by TGF-α of HER2-induced down-regulation. Conclusion: Combinatorial use of sivelestat and trastuzumab might be a novel therapeutic strategy for HER2-positive breast cancer.*

Serious inflammatory events are well known to mediate cancer growth and invasion including immediate recurrence (1). This is dependent upon the reaction of the tumor with inflammation-related cell-induced mediators (2). In the presence of inflammation, reactive cells, such as macrophages and lymphocytes, migrate to stromal tissue and secrete pro-inflammatory mediators, such as cytokines, chemokines, and prostaglandins. These mediators act on cancer cells to promote proliferation and/or angiogenesis and suppress apoptosis, resulting in tumor progression (3). Continuous inflammation converts the patient's immune status from one of predominately T-helper (Th) 1 to predominately Th2, and cytokines secreted from these inflammatory cells promote tumor cell proliferation (4). Among these pro-inflammatory mediators, neutrophil elastase (NE) released from neutrophils, well known to mediate acute lung injury and acute respiratory distress syndrome, is also thought to promote tumor growth. A unique mechanism is suggested in which NE splits epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) from the cell surface to induce activation of signal transduction in an autocrine fashion (5). Previous reports demonstrated the auto-progression of gastric and esophageal cancer cells due to NE-related growth factors (6, 7). Indeed, the prognosis of patients with elevated NE levels was reported to be poor when NE was evaluated as a predictor of prognosis (8). The control of inflammatory changes might be one of the critical steps in the regulation of cancer cell progression.

Recently, a specific synthetic NE inhibitor, sivelestat, has been developed for the treatment of various inflammatory diseases. The anti-inflammatory effect of sivelestat provides good efficacy for surgical stress, and can reduce the morbidity and organ dysfunction including acute respiratory distress syndrome.

Breast cancer with positive expression of human epithelial growth factor receptor 2 (HER2), which is detected in 25-30% of breast cancer cases, has high malignant potential that leads to poor prognosis (9). Trastuzumab, which leads to down-regulation of HER2, has improved therapy of these tumors (10), but the outcome is not always adequate, and drug intolerance is an additional factor causing worry (11). Among the drug resistance-related factors, focus has been

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placed on TGF-α, which was reported not only to inhibit HER2 disintegration by trastuzumab activation of endocytosis or lysosomes, but also to advance the recruitment of HER2 expression on the cell surface (12). TGF-α is recognized as a growth factor that increases cancer invasion and disturbs down-regulation of HER2 by anticancer agents. Inhibition of these actions of TGF-α should lead to favorable outcomes by blocking both cancer growth and drug resistance. The present study reveals the relation between NE and proliferation of breast cancer cells and whether sivelestat may both contribute and be applied to therapy for anti-HER2-positive breast cancer.

Materials and Methods

Cell line and agents. The human breast cancer cell line SKBR-3 (ATCC, Manassas, VA, USA), which is estrogen and progesterone receptor-negative and HER2-positive, was selected. Cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Sigma), 2% L-glutamine (MP Biomedicals, Eschwege, Germany), 2% sodium pyruvate (Sigma), 1% minimal essential medium non-essential amino acids solution (Sigma), 1% antibiotic/antimycotic solution (Sigma), and 0.1% tylosin solution (Sigma) in 5% CO_2 and 95% air at 37˚C.

Human NE isolated from human purulent sputum was obtained from Elastin Products Co. (Pacific, MO, USA). Sivelestat and trastuzumab were kindly provided by Ono Pharmaceutical Co. (Osaka, Japan) and Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Recombinant human TGF-α, anti-human TGF-α, and EGF antibody were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

MTT assay. Cell growth assay was assessed by a standard 3-(4,5dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay (CellTiter 96® aqueous MTT cell proliferation assay; Promega Corp., Madison, WI, USA). Following 24-h incubation in serum-free medium, i) the cells were incubated for 48 h with different concentrations of NE (1.5-25 nM), sivelestat(1-1000 μg/ml), and anti-TGF-α antibody (1-100 ng/ml), or anti-EGF antibody (0.1-10 ng/ml) in either the presence or absence of 25 nM NE; ii) the cells were incubated for 24, 48, 72, and 96 h with a 10 μg/ml concentration of trastuzumab alone or with a combination of 10 μg/ml trastuzumab and 100 μg/ml TGF-α; and iii) the cells were incubated for 48 h with 25 nM NE, 25 nM NE plus 100 μg/ml sivelestat, 25 nM NE plus 10 μg/ml trastuzumab, or 25 nM NE plus 100 μg/ml sivelestat plus 10 μg/ml trastuzumab. After incubation, the absorbance at 450/540 nm was measured using a microplate reader (BioRad, Tokyo, Japan). The absorbance of the solution from the control cells was designated as 100%. The experiments were performed in triplicate, and the mean±SD of the data were calculated. For statistical analysis of the data, Student *t*-test was used, and a value of $p<0.05$ was considered statistically significant.

Western blot analysis. Cells were incubated in serum-free medium, grown to confluence, and incubated in i) the presence of 25 nM NE alone or together with 100 μg/ml sivelestat for different periods of incubation of up to 60 min; ii) the presence of 25 nM NE alone and combinations of 25 nM NE plus 100 ng anti-TGF-α antibody, 25 nM NE plus 1000 ng anti-EGF antibody, and 25 nM NE plus 100 μg/ml sivelestat for 15 min; iii) the presence of 10 μg/ml trastuzumab alone and 10 μg/ml trastuzumab plus 100 ng/ml TGF-α for 24, 48, 72, and 96 h; and iv) the presence of 25 nM NE alone and combinations of 25 nM NE plus 100 μg/ml sivelestat, 25 nM NE plus 10 μg/ml trastuzumab, and 25 nM NE plus 100 μg/ml sivelestat plus 10 μg/ml trastuzumab for 48 h. Cells were then washed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1.0% NP_4O, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0)] containing phosphatase inhibitors (Sigma). Twenty micrograms of protein from each sample were electrophoresed through a polyacrylamide-SDS gel (Ready Gels J; Bio-Rad Laboratories, Milan, Italy) in buffer (10×Tris/glycine/SDS buffer; Bio Rad Laboratories), and blotted on polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane; Millipore, Billerica, MA, USA) in transfer buffer (10×Tris/glycine buffer; Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dried milk in TBS-T. The filters were then incubated with primary antibodies against β-actin (Cell Signaling, Boston, MA, USA), EGFR (Cell Signaling), phospho (p)-EGFR (Tyr1068; Cell Signaling), HER2 (Epitomics, Burlingame, CA, USA), pHER2 (Y877; Epitomics), extracellular signal-regulated kinase (ERK) 1/2 (Cell Signaling), and pERK1/2 (Thr202/Tyr204; Cell Signaling). Each of these antibodies was diluted as recommended by the manufacturer. Membranes were then washed with TBS-T buffer and incubated with the appropriate secondary antibodies. Immunoactivity was visualized using enhanced chemiluminescence techniques.

Results

Effect of NE on breast cancer cells. NE increased the cell growth of SKBR-3 in a dose-dependent manner as shown in Figure 1A. Cell growth was significantly increased to 108% that of the control level ($p<0.05$) with 25 nM NE, and the level continued at a plateau over 25 nM. An NE-induced signal pathway is shown in Figure 1B. NE rapidly activated EGFR and HER2 and which were then reduced to the control level after peaking at 15 min. NE also stimulated ERK1/2 phosphorylation after 5 min, and phosphorylation continued, gradually increasing over 60 min. To confirm the effect of TGF-α and EGF on cell proliferation, these antibodies were added in the absence and presence of NE (Figure 2). Both antibodies significantly inhibited cell growth induced by NE at each concentration in a dose-dependent manner, up to 90.0% with 1000 ng/ml anti-TGF-α antibody and up to 91.2% with 100 ng/ml anti-EGF antibody. Sivelestat, which is a specific inhibitor of NE, significantly blocked NE-induced cell growth at concentrations over 10 μg/ml, also in a dose-dependent manner (Figure 3A). An antibody against TGF-α or EGF reduced EGFR, HER2, and ERK phosphorylation, whereas sivelestat diminished these activations by NE completely (Figure. 3B).

Improvement of trastuzumab treatment for cancer therapy with sivelestat. As shown in Figure 4A, trastuzumab with NE resulted in notable inhibition of cell growth in comparison

with the control (92.9% at 48 h, $p=0.098$; 87.6% at 72 h, $p<0.05$; and 84.5% at 96 h, $p=0.114$), but TGF-α offset its action (113.7% at 24 h, $p<0.05$; 135.3% at 48 h, $p<0.001$; 133% at 72 h, $p<0.05$; and 118.3% at 96 h, $p<0.01$). The effect of TGF-α occurred in a dose-dependent manner with a plateau at concentrations of over 100 ng/ml (data not shown). In regard to the signaling pathway, trastuzumab clearly reduced HER2 levels after 24 h and slightly reduced EGFR levels after 96 h, whereas the presence of TGF-α blocked these actions of trastuzumab completely (Figure 4B). The effect of sivelestat on trastuzumab-mediated changes was also studied (Figure 5A). Trastuzumab and sivelestat reduced cell growth to 86.3% ($p<0.05$) and 82.1% ($p<0.001$), respectively of the control, and their combined use significantly reduced cell growth to 64.4% ($p<0.001$). In regard to the signaling pathway, sivelestat blocked the NE-suppressed action of trastuzumab on HER2 down-regulation (Figure 5B).

Discussion

Inflammation-related action is well known to lead to an unfavorable condition in patients with advanced cancer (1). NE plays a critical role (3) in several mechanisms that mediate systemic changes, especially in relation to the occurrence of cytokine storms (13). Usually detected in the treatment of gastrointestinal cancer, postoperative complications such as anastomosis leakage or severe pneumonia, promote cancer growth or immediate recurrence even after complete resection is verified macroscopically (14, 15). These clinical experiences have been indicated with NE action for autocrine fashion (16, 17). The present study is the first report, to our knowledge, to demonstrate a relation between the effects of inflammatory changes in cytokines associated with inflammation and the development of anti-HER2 therapy for breast cancer.

In the present study, NE was found to increase cell growth with EGFR, HER2, and ERK1/2 phosphorylation in breast cancer cells (Figure 1), and the cell proliferation caused by NE appeared to be related to the presence of EGF or TGF-α because antibodies for each ligand reduced the NE-induced cell growth. The ligand which activates HER2 remains unknown (18); thus, the details of HER2 phosphorylation by NE are also unclear. However, several factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), are split from the cell surface by NE (5, 6), and it is possible that HER2 is activated by cross-talk between receptors activated by these ligands and HER2. Indeed, cross-talk between insulin-like growth factor-1 receptor (IGF1R) and HER2 was demonstrated to enhance resistance to HER2-related drugs *in vitro* (18). In addition, it appears that HER2 forms dimers with other EGFR family members activated by stimulation

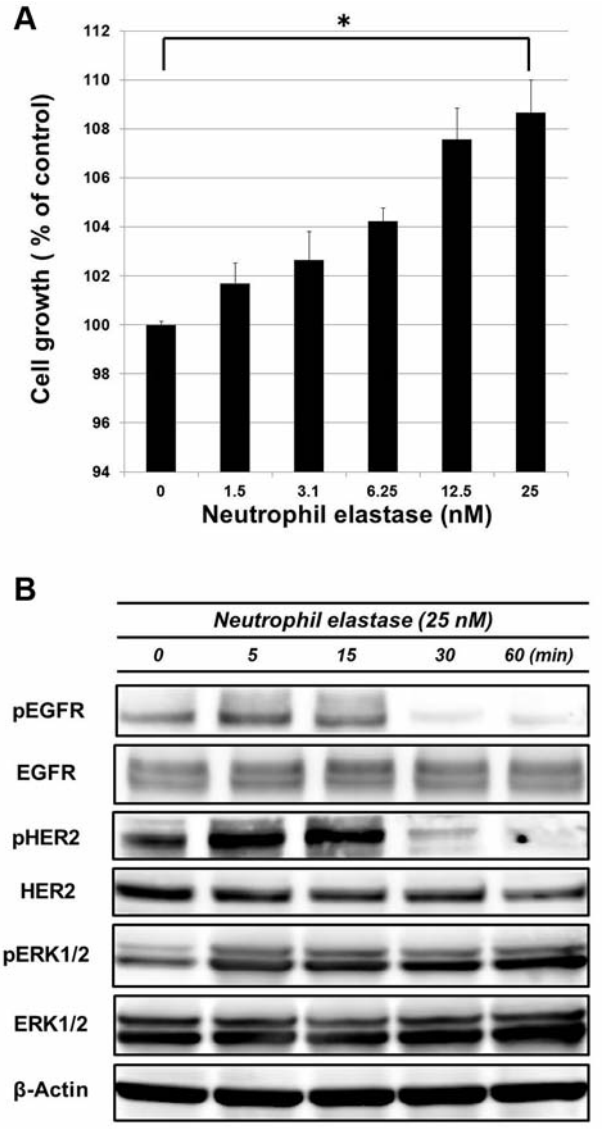

Figure 1. *The effect of neutrophil elastase (NE) on breast cancer cells. The growth of SKBR-3 cells increased in a dose-dependent manner after treatment with 25 nM NE (A). Values were obtained by MTT assay and evaluated and analyzed as described in the Materials and Methods. NE had no effect on total levels of EGFR, HER2, and ERK1/2, but a rapid initial increase in phosphorylation was observed for each (B). EGFR and HER2 activation reached their maximal levels at 30 min, and phosphorylation of ERK1/2 was continuous up to 60 min. Bands were detected by Western blot as described in the Materials and Methods. *p<0.05.*

of released ligands, and this activated receptor induces signal transduction (19). In particular, the HER2 and HER3 heterodimers have highly functional signaling units and constitute the most active signaling dimers in this family (20). In the present study, sivelestat, which blocks splitting of NE-induced growth factors from the cell surface, was found to reduce the effect of NE on cell growth through cell

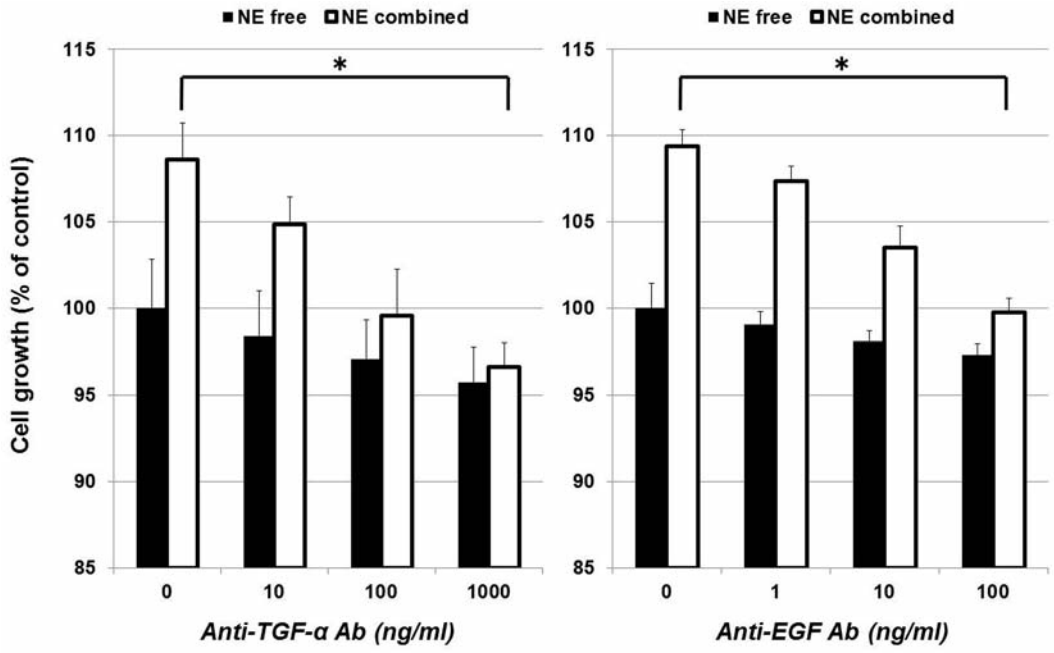

Figure 2. *The effect of TGF-α and EGF on SKBR-3 cells. The growth of cells treated with anti-TGF-α antibody and anti-EGF antibody with or without 25 nM neutrophil elastase (NE) is shown. Values were obtained by MTT assay and evaluated and analyzed as described in the Materials and Methods. *p<0.05.*

signaling pathway and EGFR and HER2 phosphorylation (Figure 3). The activation of the receptor-related cell signaling pathway was suppressed by EGF and TGF-α neutralizing antibodies but was incomplete compared with that by sivelestat, indicating that sivelestat appears to control the entire inflammatory reaction.

Trastuzumab, which was developed as a monoclonal antibody against HER2, has improved prognosis of patients with HER2-positive breast cancer, although its response rate is 15-35%, and drug resistance is usually detected in about one year (21). The response rate to second-line therapy after using trastuzumab is quite low; thus, long-term treatment with trastuzumab is expected (22, 23). The mechanisms of resistance to trastuzumab have mainly been shown to be obstacles in binding (24), up-regulation of HER2 downstream signaling pathways (25) and an alternate pathway of signal transduction (26). In addition, a recent study focused on an action of TGF-α to impair HER2 down-regulation (12). TGF-α has been found not only to suppress HER2 down-regulation by disrupting endocytosis and lysosome function, but also to recruit HER2 on the cell surface (27). Indeed, clinical experience shows that patient prognosis with HER2 therapy is significantly poorer if TGF-α expression is high in serum or tumor tissue (28, 29). The present study showed that TGF-α disturbs the trastuzumab-induced growth inhibitory effect through HER2 down-regulation. In addition, NE also reduced the trastuzumab-mediated response similarly to the action of TGF-α. Thus, to control cancer cell growth more completely, it might be critical to simultaneously suppress actuation of multiple HER family members because EGFR inhibition is already known to increase HER2 activation (30). In recent clinical experience with cancer therapy focusing on combination treatment with trastuzumab and sivelestat, despite the fact that antibodies for EGFR and/or HER2 reduced NE-induced activation of each receptor, the presence of a suppressor for both receptors was more important (31). A recent report from a clinical trial showed higher expression of NE to be detected in trastuzumab-responsive cancer tissue (32), suggesting that as well as its relation to TGF-α, NE is also related to other still unknown factors. This might be a critical time to argue for combination therapy with trastuzumab. Furthermore, as another reason for the efficacy of such combination therapy, sivelestat suppresses signal transduction from HER2/HER3 or EGFR/HER2 because trastuzumab more intensely affects HER2 homodimers but does not affect the EGFR/HER2 or HER2/HER3 heterodimers influenced by TGF-α or EGF, which have an intensive ability for signal transduction (20).

Conclusion

In the past decade of breast cancer therapy, treatment of HER2-positive cancer has been problematic because of its high potential for malignancy and drug resistance. In

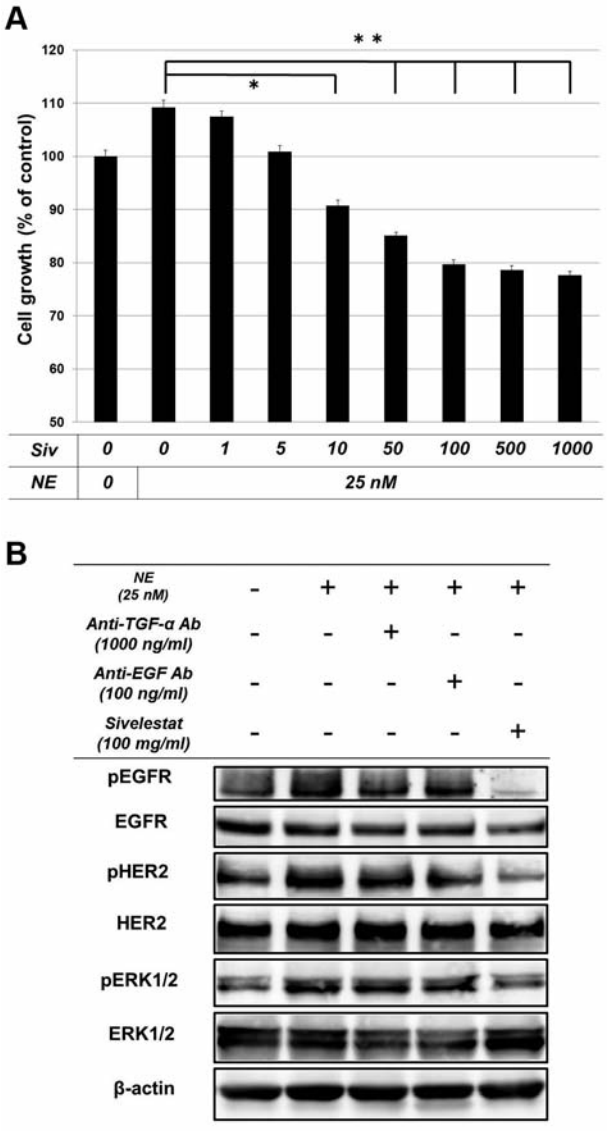

Figure 3. *Cell growth suppression by sivelestat. A growth inhibitory effect of sivelestat (Siv) was noted in a dose-dependent manner (A). Values were obtained by MTT assay and evaluated and analyzed as described in the Material and Methods. Sivelestat markedly suppressed the phosphorylation of EGFR, HER2, and ERK1/2 by neutrophil elastase (NE) (B). The antibody for TGF-α or EGF was added 15 min before the addition of 25 nM NE. Bands were detected 15 min after addition of antibodies by Western blot method as described in the Materials and Methods. *p<0.05, **p<0.01.*

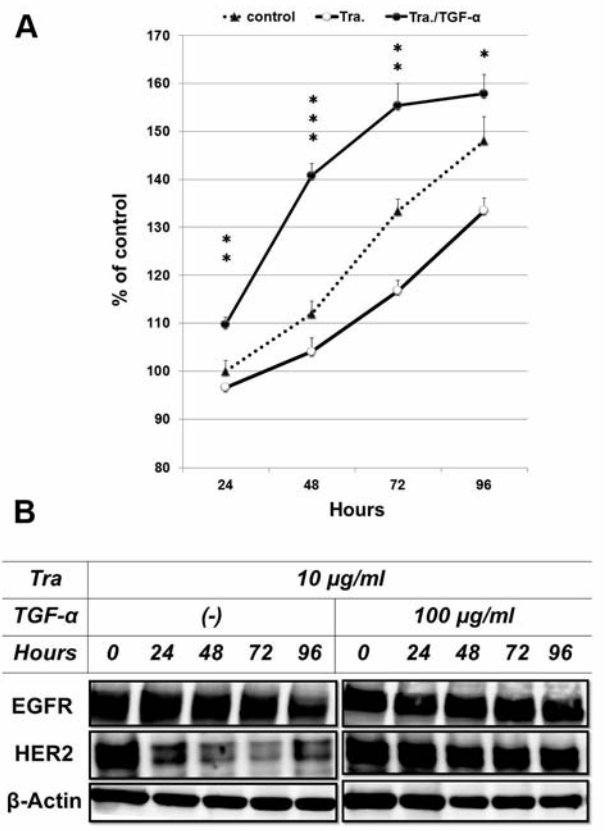

Figure 4. *Suppression of trastuzumab-induced effect by TGF-α. Trastuzumab(Tra) at 10 μg/ml inhibited cell growth compared with control, but TGF-α 100 μg/ml suppressed the effect of trastuzumab (A). Cells were incubated for 24-96 h with each factor, and the values were obtained by MTT assy. Trastuzumab at 10 μg/ml induced down-regulation of HER2 from 24 hours and EGFR from 96 hours, but TGF-α 100 μg/ml suppressed this effect of trastuzumab (B). Trastuzumab was added 15 min before TGF-α. Bands were detected at 0-96 hours by Western blot method as described in the Materials and Methods. *p<0.05, **p<0.01, ***p<0.001.*

addition to evidence that TGF-α plays an important role in the disturbance of HER2 targeting therapy, the present study revealed the benefit of a possible novel combination of trastuzumab with sivelestat. The combination of these two drugs may open the door to a new decade of therapy for HER2-positive breast cancer.

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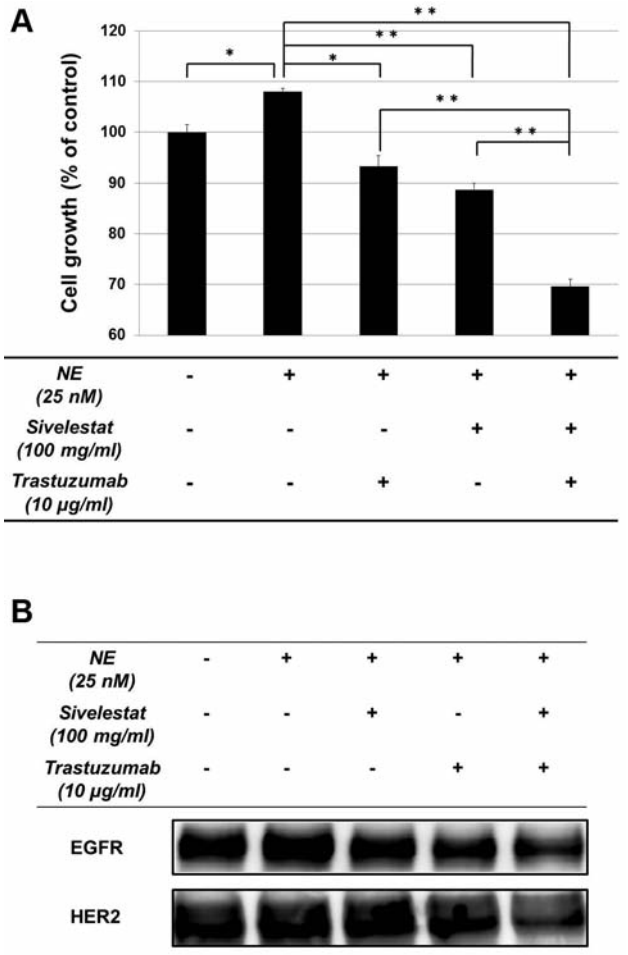

Figure 5. *Effect of sivelestat on trastuzumab resistance. Combination use of sivelestat and trastuzumab demonstrated more intensive cell growth inhibition than the use of each agent alone (A). Cells were incubated for 48 h with each agent, and the values were obtained by MTT assay. HER2 down-regulation induced by trastuzumab at 10 μg/ml was blocked by the presence of 25 nM neutrophil elastase (NE), and but restored by sivelestat at 100 mg/ml (B). Sivelestat at 100 mg/ml was added 15 min before trastuzumab and NE. Bands were detected at 48 h by Western blot method as described in the Materials and Methods. *p<0.05, **p<0.01.*

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