Abstract. Our study used protein array technology to analyze the expression status of various activated receptor tyrosine kinases (RTKs) in gastric carcinoma; then, we sought to discover an effective therapeutic receptor tyrosine kinase for this disease and investigated the anti-tumor mechanism of the therapeutic RTK. In addition to the expressions of activated RTKs in human gastric cancer and adjacent normal mucosa, the expression of activated RTKs in gastric cancer cell lines, MKN74, MKN45, MKN7 and MKN1, were also studied. The RTKs activated in gastric cancer tissue are EGFR, ErbB2, FGFR1, FGFR2, insulin R, and EphA4. Among the RTKs activated in gastric cancer tissues, EGFR and ErbB2 were also activated in all gastric cell lines examined in this study. A subsequent in vitro experiment using subcutaneous gastric cancer-bearing athymic nude mice demonstrated that the ErbB2-targeting drug trastuzumab markedly suppressed the growth of gastric cancer. Moreover, using an angiogenesis protein array, the expressions of Ang I, FGF-ß, TGF-ß and IL-8 in MKN74 xenograft tumors were found to be significantly reduced by treatment with trastuzumab, indicating that trastuzumab may inhibit the expression of angiogenic molecules in MKN74 cells in vivo. These data suggest that ErbB2 is activated in gastric cancer, and the ErbB2-targeting drug trastuzumab may be related to the reduction of Ang 1, FGFα, FGFβ, TGFα and IL-8.

Introduction

Gastric cancer is now the second-leading cause of cancer-related mortality worldwide (1). Apart from potentially curative surgery, chemotherapy and radiochemotherapy may be applied at advanced stages in gastric cancer, but do not cure the disease in such cases, and the prognosis is poor. Thus, there is a strong demand for new curative approaches to gastric cancer therapy.

Receptor tyrosine kinases (RTKs) are a family of 56 proteins each characterized by a transmembrane domain and a tyrosine kinase motif (2). The known RTKs consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein kinase activity, are divided into 21 families, such as epidermal growth factor (EGFR), vascular endothelial growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR), characterized by a similar structure and the potential of intrafamilial dimerization (3). Various RTKs have been implicated in intracellular signal transduction including growth, differentiation, adhesion, migration, apoptosis and carcinogenesis (4). Aberrant RTK activity was initially found in various epithelial cancers, such as breast cancer, hepatocellular carcinoma, and lung cancer, as well as gastric cancer. It is considered that receptor tyrosine kinases play an important role in the development of almost all types of cancer (5-7). As a result, several clinical trials are exploring in different settings and with diverse designs the potential of anti-RTK therapies in various cancer types. However, the mechanism of the suppression of cancer growth by RTK-targeting drugs remains relatively unknown.

Here we have investigated the various RTKs that are activated in gastric cancer using protein arrays. We have shown that the immunological inhibitor for ErbB2, trastuzumab, is effective in gastric cancer, and have also examined the relationship between the growth suppression of trastuzumab and the expression of angiogenic molecules to explore the mechanism of the anti-tumor effect of trastuzumab.

Materials and methods

Materials. RayBio™Human Phospho Array Kit (Catalog no. ARY 001) was purchased from RayBiotech, Inc. (Norcross, GA, USA). TranSignal™ Angiogenesis Antibody Array was purchased from Panomics, Inc. (Fremont, CA, USA).
Plated at the density of $1 \times 10^5$ cell/cm$^2$ in plastic flasks of were used as the gastric cancer cell lines. These cells were gift of the Japanese Cancer Resource Bank (Tokyo, Japan),

Committee of Kagawa University School of Medicine.

Cell line. MKN74, MKN45, MKN7, and MKN1 cells, a kind gift of the Japanese Cancer Resource Bank (Tokyo, Japan), were used as the gastric cancer cell lines. These cells were plated at the density of $1 \times 10^5$ cell/cm$^2$ in plastic flasks of Dulbecco's modified minimum essential medium (DMEM) (Gibco BRL Co., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 μg/ml) and 100 μg/ml streptomycin at 37°C in 5% CO$_2$ in air.

Cell lysate and tissue lysate. The lysate was performed according to the methods described in our previous reports (8-10). All steps were carried out at 4°C. Protein concentration was measured using a dye-binding protein assay based on the Bradford method.

Antibody arrays of phospho-RTK. An assay for phospho-RTK array was performed according to the manufacturer's instructions. Briefly, phospho-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h. Membranes were then incubated with ~2 ml of lysate prepared from cell lines or tissues after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% Tween-20, 3 times for 10 min, and TBS alone, 2 times for 10 min, to remove unbound materials, the membranes were then incubated with anti-phospho-tyrosine-HRP antibody for 2 h at room temperature. The unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Amersham Life Sciences, Tokyo, Japan).

Angiogenesis antibody array. To assess whether molecules important in angiogenesis are regulated by the anti-tumor effect of trastuzumab, the RayBio Human Angiogenesis Antibody Array (RayBiotech, Inc., GA) was used according to the protocol. This method is a dot-blot based assay which enables detection and comparison of 19 different angiogenesis-specific cytokines. Briefly, angiogenesis protein array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h. Membranes were then incubated with ~2 ml of lysate prepared from tumor tissues after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% Tween-20, 3 times for 5 min, to remove unbound materials, the membranes were then incubated with HRP angiogenesis antibody for 2 h at room temperature. Unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Amersham Life Sciences).

In vivo anti-tumor effects of trastuzumab on gastric cancer. Athymic 8-week-old male BALB/c/nu/nu mice, weighing 20-22 g, were purchased from Japan SL(C Hamamatsu, Japan) and kept under specific pathogen-free conditions at 24±2°C. Animal experiments were performed with approved protocols and in accordance with the institutional recommendations for the proper care and use of laboratory animals. MKN human gastric cancer cells were suspended in PBS at a concentration of $5 \times 10^7$ cells/ml, and 100 μl inoculum volumes were injected subcutaneously into the flank regions of athymic BALB/c/nu/nu mice. When the tumor became palpable in the treated group (n=10), 500 μl of PBS containing 750 mg/0.5 ml trastuzumab (Herceptin, directed against the erbB2 receptor, also known as Her2/Neu oncogene), was administered intraperitoneally for 3 weeks, 3 times a week. Only PBS was administered to the control group (n=10). After the initiation of the administration of trastuzumab, the tumor growth was monitored by the same investigators (J. Gong and T. Masaki), and the tumor diameters were measured every week using a graduated caliper. Tumor growth was assessed weekly by measuring the two greatest perpendicular tumor dimensions. Tumor volume was calculated as follows: tumor volume (mm$^3$) = [tumor length (mm) x tumor width (mm)]/2 (11). All animals were sacrificed on day 24 after treatment. All animals were alive during the observation.

Statistical analysis. Results are expressed as mean ± SD. All analyses were performed using the computer-assisted StatView program (SAS Institute, Gray, NC, USA). Paired analysis between two groups was performed using the t-test. A p=0.05 was considered to indicate a significant difference between groups.

Results

The activity level of tyrosine-activated receptor tyrosine kinases (RTKs) associated with gastric carcinoma. We used a phospho-RTK array system to identify the 'key RTKs' associated with gastric carcinoma (Fig. 1). By using the antibody array, we simultaneously screened the expressions of 42 different activated RTKs (Fig. 1). The RTKs activated in gastric cancerous tissue were EGFR, ErbB2, FGFR1, FGFR2α, insulin R and EphA4 (Fig. 2A). Compared with their levels in normal mucosa, these activated RTKs were upregulated in all cancerous samples (5 cases) used in this study. Among these activated RTKs in gastric cancerous tissue, ErbB2 was also activated in all of the four gastric cancer cell lines, namely, MKN45, MKN74, MKN1 and MKN7 (Fig. 2B). These results suggest that an ErbB2-targeting drug may be a useful agent for the treatment of gastric cancer.

In vivo anti-tumor effects of ErbB2-targeting drug, trastuzumab. Athymic 8-week-old male BALB/c/nu/nu mice were implanted subcutaneously with MKN74. When the animal developed palpable tumors, they were treated intraperitoneally for 3 weeks, 3 times a week. Animals in the control group received intra-peritoneal administration of the vehicle (PBS). As shown in Fig 3A, animals in the control group developed rapidly growing subcutaneous gastric cancer. In contrast, animals in the trastuzumab groups exhibited significantly
retarded tumor development compared with animals in the control group (Fig. 3A). Fig. 3B (a and b) are representative photographs of the gross MKN74 tumors from nude mice treated with either trastuzumab or control, respectively. Furthermore, animals in the trastuzumab group did not show any apparent changes, while those in the control group showed disheveled fur and body weight. All animals were alive during the experiment.

As shown in Fig. 3C, both ErbB2 and ErbB4 in MKN74 tumors of nude mice treated with trastuzumab (Fig. 3C,a) were not activated compared with control nude mice (Fig. 3C,b). The activity level of ErbB2 and ErbB4 in trastuzumab-treated nude mice was 4.36 and 20.67% of the control, respectively (Fig. 3D).

Expression of various angiogenesis antigens in MKN74 tumor tissues. Using various angiogenic protein arrays (Fig. 4A), the expressions of FGF-α, FGF-β Ang 1, TGF-α, and IL-8 in the tumor tissue were reduced by trastuzumab (Fig. 4B). The expression ratios of Ang 1, FGF-α, FGF-β, IL-8, and TGF-α were 0.40, 43.87, 49.71, 57.47, and 18.21% of the control, respectively (Fig. 4C).

Discussion

ErbB-2 (also known as HER-2) encodes a receptor tyrosine kinase which plays a role as a growth regulatory protein and a cell motility factor (12). Overexpression of ErbB-2 is frequently observed in a variety of tumors (13-18). In gastric carcinoma, overexpression and/or amplification of ErbB-2 has been reported at a 6-30% incidence (19-21). These studies suggest that overexpression of ErbB-2 has been associated with poor survival in gastric carcinoma. On the other hand, in experimental models, trastuzumab suppresses the growth of
human gastric cancer with HER2 overexpression in vitro and in vivo (22-27). As a result of these preclinical data, several clinical trials are exploring in different settings and with diverse designs the potential of anti-HER2 therapies in gastric cancer patients (22,23,26). However, to date, the mechanism of the growth suppression of trastuzumab for gastric cancer cells remains unknown. In the present study, we examined the relationship between the anti-tumor effect of trastuzumab and the expressions of angiogenic molecules.

Using the protein array, the activations of EGFR, ErbB2, FGFR1, and FGFR2 were detected in all gastric cancer tissues and cell lines studied. EphA4 was activated in all gastric cancer tissues and other cell lines except MKN1 used in the present study. Overexpression of EGFR, ErbB2, FGFR1, FGFR2, EphA4 has been already reported in gastric cancer (26,28-31). The previous report support our results on the various RTKs activated in gastric cancer derived from the protein array used in this study. In summary, the use of protein array in this study suggests that it may be a useful tool for studying the expression of activated RTKs in various tissues, including malignant tissues. Furthermore, these results suggest that the immunological
inhibition of EGFR, ErbB2, FGFR1, FGFR2α, and EphA4 may have an anti-tumor effect for gastric cancer.

Some studies have reported that trastuzumab directed against ErbB2, among the enhanced RTKs in gastric cancer, is effective in patients with gastric cancer (22,23). However, the anti-tumor mechanism for trastuzumab has remained relatively unknown. To date, there are two anti-tumor mechanisms proposed for the therapeutic effect of trastuzumab: a direct anti-proliferative effect via a blockade of signaling pathways, down-modulation of the ErbB2 protein, and activation of apoptotic signals of the tumor cells, and an indirect anti-tumor effect by antibody-dependent cell-mediated cytotoxicity activity (32,33). In the present study, the expression of various angiogenic molecules, such as Ang 1, FGF-α, FGF-β, IL-8 and TGF-β in the tumor tissue of trastuzumab treated nude mice were 0.40, 43.87, 49.71, 57.47 and 18.21% of the control, respectively.

In conclusion, our findings demonstrate that the expressions of Ang 1, FGF-α, FGF-β, IL-8 and TGF-β in gastric cancer
are regulated by ErbB2 activity, and they suggest an additional mechanism for the contribution of the ErbB2-targeting drug trastuzumab to the inhibition of tumor angiogenesis.

References