Abstract  Purpose: A malfunction of retinoid X receptor-α (RXRα) due to phosphorylation by the Ras/mitogen-activated protein kinase signaling pathway is associated with the development of hepatocellular carcinoma (HCC). The humanized anti-HER2 monoclonal antibody trastuzumab inhibits the activation of HER2 and its multiple downstream signaling pathways, including the Ras/mitogen-activated protein kinase pathway. In this study, the effects of phosphorylation of RXRα on the ability of RXRα ligand 9-cis-retinoic acid (9cRA) and trastuzumab to inhibit growth of HCC cells was examined.

Experimental Design: The effects of a combination of 9cRA plus trastuzumab on the inhibition of cell growth in HLF human HCC cells which express constitutive activation of HER2 protein were examined.

Results: The combination of 9cRA plus trastuzumab synergistically inhibited the growth of HLF cells without affecting the growth of Hc normal human hepatocytes. Combined treatment with these agents acted synergistically to induce apoptosis in HLF cells. The treatment of HLF cells with trastuzumab alone inhibited the phosphorylation of HER2, RXRα, ERK, Akt, and Stat3 proteins and these effects were enhanced when the cells were cotreated with 9cRA. Reporter assays indicated that the combination of 9cRA plus trastuzumab markedly increased both the retinoic acid responsive element and retinoid X responsive element promoter activities in HLF cells.

Conclusion: 9cRA and trastuzumab cooperatively inhibit the activation of HER2 and its downstream signaling pathways, subsequently inhibiting the phosphorylation of RXRα and the growth of HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

Hepatocellular carcinoma (HCC) is a serious healthcare problem worldwide. Prevention of HCC in viral liver cirrhosis has recently received increasing attention because the incidence and recurrence rate of this cancer in those patients is very high (1). One of the promising agents for HCC chemoprevention is retinoids (2). Retinoids, a group of structural and functional analogues of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development (3, 4). Retinoids exert their biological functions primarily by regulating gene expression through two distinct nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR), which are both composed of three subtypes (α, β, and γ). Among these receptors, RXRα acts as a master regulator of nuclear receptors and controls normal cell proliferation and metabolism (3, 4).

Because of such physiologic functions of retinoids and their receptors, abnormalities in the expression and function of these molecules are highly associated with the development of various cancers, including HCC. We have previously shown that hepatocarcinogenesis is accompanied by an accumulation of the phosphorylated (i.e., inactivated) form of RXRα protein (p-RXRα; ref. 5). The phosphorylation at serine 260 of RXRα, a consensus site of mitogen-activated protein kinase (MAPK), abolishes its ability to form heterodimers with RARβ, leading to uncontrolled cell growth (6, 7). Moreover, the abrogation of phosphorylation by MAPK-specific inhibitors restores the degradation of RXRα in a ligand, 9-cis-retinoic acid (9cRA)–dependent manner, and induces apoptosis and inhibits the growth of HCC cells (6). Therefore, the inhibition of RXRα phosphorylation by targeting its upstream signaling pathways may be an effective strategy for inhibiting the growth of HCC cells.

The Ras/MAPK pathway is one of the major components of the signaling pathways that transmit mitogenic signal from the receptor tyrosine kinases (RTK; refs. 8, 9). Epidermal growth factor receptor (EGFR), HER2, and HER3 belong to subclass I of the RTK superfamily (8, 9). Abnormalities in the expression and function of RTKs play a critical role in the development of human malignancies and are thereby regarded as a promising target for the treatment of various types of malignancies,
including HCC (10, 11). Among the RTK-targeted agents, the humanized anti-HER2 antibody trastuzumab (Herceptin) is widely used for the treatment of HER2-overexpressing breast cancers (12, 13). Trastuzumab induces HER2 receptor down-modulation, and as a result, inhibits critical signaling pathways, including Ras/MAPK and phosphatidylinositol 3-kinase/Akt pathways (12, 13). In view of these observations, there has been considerable interest in using the combination of 9cRA and trastuzumab for the prevention and treatment of HCC. The aim of this study is to investigate whether the combination of 9cRA plus trastuzumab exerts synergistic growth-inhibitory effects on human HCC cells and to examine possible mechanisms for such synergy, predominantly focusing on the inhibitory effects on RXRa phosphorylation by the combination of these agents.

Materials and Methods

**Materials.** 9cRA was purchased from Sigma Chemical, Co. Trastuzumab was from CHUGAI Pharmaceutical Co., Ltd. Anti-RXRa antibody was from Santa Cruz Biotechnology. Anti–extracellular signal-regulated kinase (ERK), anti–phosphorylated ERK, anti–Akt, anti–phosphorylated Akt, anti–Stat3, and anti–phosphorylated Stat3 antibodies were from Cell Signaling Technology. Antibody against glyceraldehyde-3-phosphate dehydrogenase was from Chemicon International. DMEM and FCS were from Invitrogen. CS-C complete medium was from Cell Systems Biotechnologie Vertrieb GmbH.

**Cell lines and cell culture.** Six human HCC cell lines, HLF, PLC/PRF/5, HepG2, HuH7, HLE, and Hep3B were obtained from the Japanese Cancer Research Resources Bank and were maintained in DMEM supplemented with 10% FCS. Hc human normal hepatocyte cell line was purchased from Applied Cell Biology Research Institute and was maintained in CS-C complete medium. The cells were cultured in an incubator with humidified air and 5% CO2 at 37°C.

**Protein extraction and Western blot analysis.** Total cellular protein was extracted and equivalent amounts of protein were examined by Western blot analysis using specific antibodies, as previously described (6). For detection of the expression level of p-RXRa protein, RXRa protein was affinity-purified from the total cell extracts using anti-RXRa antibody–immobilized Sepharose beads and then was subjected to Western blot analysis using an antiphosphoserine antibody (6). Glyceraldehyde-3-phosphate dehydrogenase expression served as a loading control.

**Cell proliferation assays.** Three thousand HLF or Hc cells were seeded into 96-well plates. The following day, the indicated concentrations of 9cRA or trastuzumab were added to each well and the cells were incubated for an additional 3 days. The number of viable cells in replica plates were then counted using Trypan blue dye exclusion method, as
previously described (14). To determine whether the combined effects of 9cRA plus trastuzumab were synergistic, HLF cells were treated with the indicated concentrations of 9cRA alone, trastuzumab alone, and various combinations of these agents for 3 days. The number of viable cells in replica plates were then counted using Trypan blue dye exclusion method and expressed as a percentage of the control value. A, 9cRA alone (●); 9cRA + 5 μg/mL trastuzumab (●); 9cRA + 10 μg/mL trastuzumab (▲); 9cRA + 20 μg/mL trastuzumab (*). B, trastuzumab alone (●); trastuzumab + 5 μmol/L 9cRA (■); trastuzumab + 10 μmol/L 9cRA (▲); trastuzumab + 20 μmol/L 9cRA (*). Bars, SD of triplicate assays. C, the data obtained in A and B were used to calculate the combination index, as described in Materials and Methods. D, effects of sequential treatment with 9cRA and trastuzumab on the proliferation of HLF cells. The cells were cultured for an initial 24 h in the presence of vehicle, 5 μmol/L of 9cRA, or 10 μg/mL of trastuzumab and subsequently incubated for another 24 h with one of these agents. Cell number was counted using the Trypan blue dye exclusion method and expressed as a percentage of vehicle-treated cells (column 1). Columns, mean; bars, SD. Similar results were obtained in a repeat experiment. *P < 0.05; **P < 0.01, compared with vehicle-treated cells (column 1); +, P < 0.05, compared with column 6.

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assays. HLF cells were treated with 5 μmol/L of 9cRA alone, 10 μg/mL of trastuzumab alone, or the combination of these agents for 48 h on coverslips. The cells were then fixed with 3.7% formaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) methods using In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics), as described previously (14).

Retinoic acid responsive element and retinoid X responsive element reporter assays. Reporter assays were done as described previously (6). HLF cells were transfected with retinoid X responsive element (RXRE) or retinoic acid responsive element (RARE) reporter plasmids (750 ng/35 mm dish), which were provided by the late Dr. K. Umesono (Kyoto University, Kyoto, Japan), along with pRL-CMV (Renilla luciferase, 100 ng/35 mm dish, Promega) as an internal standard to normalize the transfection efficiency. Transfections were done using UniFector reagent (B-Bridge) according to the manufacturer’s protocol. After exposure of the cells to the transfection mixture for 24 h, the cells were treated with 5 μmol/L of 9cRA alone, 10 μg/mL of trastuzumab alone, or the combination of these agents for 24 h. The cell lysates were then prepared and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega), as previously described (6).

Statistical analysis. The data are expressed as mean and SD. Statistical significance of the difference in mean values was assessed with one-way ANOVA, followed by Sheffe’s t test.

Results

Expression of signaling components downstream of EGFR, HER2, and HER3 in HCC cells. We initially examined whether EGFR, HER2, and HER3 proteins and their downstream signaling molecules ERK and Akt proteins were overexpressed and constitutively activated in HLF, PLC/PRF/5, HepG2, HuH7,
HLE, and Hep3B human HCC cell lines and in the Hc human normal hepatocyte line using Western blot analysis. Among these HCC cell lines, HLF markedly expressed EGFR and HER2 when compared with the Hc cell line (Fig. 1). The phosphorylated (i.e., activated) form of HER2 (p-HER2) and HER3 (p-HER3) were also increased in the HLF cell line, although p-EGFR was not detected in all series of HCC cell lines and Hc cell line. These findings are consistent with those of a previous report (16) and are interesting because HER2 is the preferred heterodimerization partner for the other members of the EGFR family of RTKs and HER2/HER3 heterodimer can play a critical role in allowing the efficient activation of potent oncogenic signaling cascades (17, 18). In the HLF cell line, there was also a marked increase in the expression levels of p-ERK and p-Akt proteins, which induce the aberrant phosphorylation of RXRα protein and therefore play a role in the development of HCC (19). Therefore, the following combination studies using 9cRA plus trastuzumab were done using this cell line.

9cRA plus trastuzumab causes preferential inhibition of growth in HLF human HCC cells in comparison with Hc normal hepatocytes. We next examined the growth-inhibitory effect of 9cRA and trastuzumab on HLF and Hc cell lines. As shown in Fig. 2, 9cRA and trastuzumab inhibited the growth of HLF cell lines with an IC50 value of ~27 μmol/L and 22 μg/mL, respectively. The Hc normal human hepatocytes were more resistant to these agents because the IC50 values with both compounds were ~100 μmol/L and 100 μg/mL, respectively. These results suggest that 9cRA and trastuzumab preferentially inhibit the growth of HLF cells (Fig. 2).

9cRA plus trastuzumab causes synergistic inhibition of growth in HLF cells. We previously reported the combined treatment of 9cRA plus MAPK inhibitor to inhibit the proliferation of HCC cells and this was associated with a degradation of p-RXRA protein (6). Therefore, the ability of the combined treatment with a range of concentrations of 9cRA plus trastuzumab to synergistically inhibit the growth of HCC cells was investigated because trastuzumab targets HER2 receptor and inhibits the activation of downstream molecules ERK and Akt proteins (12, 13). We found that the combination of as little as 5 μmol/L of 9cRA and 10 μg/mL of trastuzumab (the approximate IC50 values for both compounds) exerted synergistic growth inhibition (Fig. 3A and B). The isobologram analysis (15) gave a combination index of 2+ to this mixture (Fig. 3C; Table 1). These findings suggest that 9cRA plus trastuzumab might be an effective combination for the inhibition of HCC cell growth due to their synergistic efficacy.

**Table 1. Combined effects of 9cRA and trastuzumab on HLF cells**

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Abbreviations: CI, combination index; "±", CI 0.9-1.1 additive effect; "+", CI 0.8-0.9 slight synergism; "++", CI 0.6-0.8 moderate synergism; "+++", CI 0.4-0.6 synergism; "++++", CI 0.2-0.4 strong synergism.

We next examined whether 9cRA might enhance the growth-inhibitory effect of trastuzumab or, alternatively, whether trastuzumab might amplify the antiproliferative effect of 9cRA (Fig. 3D). HLF cells were first exposed to either 5 μmol/L of 9cRA or 10 μg/mL of trastuzumab for 24 hours, followed by incubation with the second respective agent for an additional 24 hours. The most significant reduction in the number of viable cells was found when 9cRA was administrated following trastuzumab treatment (Fig. 3D, column 7). These results suggested that trastuzumab might enhance the sensitivity of the cancer cells to 9cRA.

9cRA plus trastuzumab causes a synergistic decrease in the phosphorylated forms of HER2, ERK, Akt, and Stat3 proteins in HLF cells. We next examined whether the synergistic growth inhibition by the combined treatment of 9cRA plus trastuzumab (Fig. 3C; Table 1) was associated with the inhibition of RXRα phosphorylation. Treatment with 10 μg/mL of trastuzumab alone caused a decrease in the expression levels of p-HER2, p-ERK, p-Akt, p-Stat3, and p-RXRA proteins in HLF cells (Fig. 4, column 3). There was also a decrease in the expression levels of p-HER2, p-ERK, p-Akt, and p-Stat3 proteins when the cells were treated with 5 μmol/L of 9cRA alone (Fig. 4, column 2). Moreover, the decreases in the expression of p-RXRA, p-ERK, p-Akt, and p-Stat3 proteins was
9cRA plus trastuzumab synergistically induces apoptosis in HLF cells. We then examined whether the synergistic growth inhibition by combined treatment with 9cRA plus trastuzumab (Fig. 3C; Table 1) might be explained by the induction of apoptosis because both of these agents could induce apoptosis in cancer cells (4, 13). The treatment of HLF cells with either 5 μmol/L of 9cRA alone or 10 μg/mL of trastuzumab alone induced terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells in ~10% of the total remaining cells (Fig. 5, columns 2 and 3). Moreover, the combination of these agents markedly enhanced the induction of apoptosis in 39% of the total remaining cells (Fig. 5, column 4). These results strongly suggest the synergism in inducing apoptosis by the combination of 9cRA and trastuzumab.

Trastuzumab enhances the induction of both RARE and RXRE promoter activities by 9cRA. RARs and RXRs modulate the expression of target genes by interacting with RARE or RXRE elements located in the promoter regions of these genes (3, 4). We conducted transient transfection luciferase reporter assays to determine whether trastuzumab might enhance the induction of RARE or RXRE promoter activities by 9cRA. We found that 5 μmol/L of 9cRA alone significantly increased both RARE and RXRE reporter activities (Fig. 6A and B, columns 3). Moreover, when trastuzumab was combined with 9cRA, there was a synergistic increase in the transcriptional activity of these reporter activities (Fig. 6A and B, columns 4).

Discussion

The present study showed that the combination of 9cRA plus trastuzumab synergistically inhibited the growth of HLF human HCC cells (Fig. 3; Table 1) and that this was associated with the induction of apoptosis (Fig. 5). A hypothetical scheme which explains the mechanism is proposed in Fig. 7. In normal hepatocytes, ligand-activated (9cRA) RXRs forms a heterodimer with other nuclear receptors and binds to the specific responsive element in the promoter region of target genes (3, 4). However, in HCC cells, Ras/MAPK is highly activated and phosphorylates RXRα. The accumulated nonfunctional p-RXRα interferes with the function of the remaining normal RXRs in a dominant-negative manner, thereby promoting the growth of HCC cells (6). Therefore, p-RXRα and its kinases may

Fig. 5. Effects of the combination of 9cRA plus trastuzumab on the induction of apoptosis in HLF cells. The cells were treated with vehicle (column 1), 5 μmol/L of 9cRA alone (column 2), 10 μg/mL of trastuzumab alone (column 3), or the combination of 5 μmol/L of 9cRA plus 10 μg/mL of trastuzumab (column 4) for 48 h. The cells were then stained using terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling method. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells were counted and expressed as the percentage of total cell number (500 cells were counted in each flask). Columns, mean; bars, SD; *, P < 0.05; **, P < 0.01, compared with vehicle-treated cells (column 1). Representative results from three independent experiments with similar results.

Fig. 6. Effects of the combination of 9cRA plus trastuzumab on transcriptional activity of RARE (A) and RXRE (B) promoters in HLF cells. Transient transfection reporter assays were done with the indicated luciferase reporter in the presence of vehicle (column 1), 10 μg/mL of trastuzumab alone (column 2), 5 μmol/L of 9cRA alone (column 3), or the combination of 10 μg/mL of trastuzumab plus 5 μmol/L of 9cRA (column 4). Relative luciferase activity was determined after 24 h. Columns, mean; bars, SD; *, P < 0.05; **, P < 0.01, compared with vehicle-treated cells (column 1).
be useful targets for inhibiting the growth of HCC cells. RTKs are one of the potent activators for Ras/MAPK (8, 9), and we showed that trastuzumab inhibited the activation of HER2 and its downstream ERK, Akt, and Stat3 proteins, thus inhibiting the phosphorylation of RXRα protein (Fig. 4). These new findings suggest that trastuzumab cooperatively restores the function of RXRα as a master regulator of nuclear receptors in HCC cells and this might be associated with the preferential inhibition of growth in HCC cells in comparison to normal hepatocytes (Fig. 2). In this context, the order of administration is important for such synergism, as shown in Fig. 3.

The advantageous effects of the combination of retinoids plus trastuzumab have been reported in breast cancer cells. Specifically, HER2-overexpressing breast cancer cells were resistant to retinoid-induced growth inhibition and apoptosis, but the inhibition of HER2 by trastuzumab resulted in the recovery of RARE activity and reduced Akt activity, thus, resensitizing these cells to retinoids (20, 21). These reports are consistent with our findings that trastuzumab inhibited the activation of Akt (Fig. 4) and enhanced the activation of RARE and RXRE promoters by 9cRA in HER2-overexpressing HLF human HCC cells (Fig. 6). As to the concentration of the compounds, the plasma level of trastuzumab in clinical trials ranged from 50 to 240 μg/mL (22). Thus, the dosage that we used in the present study (10 μg/mL) is well within the practical level. Regarding retinoid dosage, clinical information is available for acyclic retinoid, a RXRα-ligand similar to 9cRA, which has been given at 1 to 5 μmol/L in patients with HCC (2). This concentration of both 9cRA (Figs. 3–6; Table 1) and acyclic retinoid (data not shown) exerted synergism with trastuzumab. Moreover, this study also showed that 9cRA alone inhibited the phosphorylation of RXRα protein (Fig. 4). These new findings suggest that trastuzumab cooperatively restores the function of RXRα as a master regulator of nuclear receptors in HCC cells and this might be associated with the preferential inhibition of growth in HCC cells in comparison to normal hepatocytes (Fig. 2). In this context, the order of administration is important for such synergism, as shown in Fig. 3.

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As to the practical application of the synergistic combination of trastuzumab and retinoids, HER2 expression is necessary because of the mechanism of action as proposed in Fig. 7. Thus, the indication of this combination might be limited because only HLF had an elevated HER2 expression among the cell lines examined in this study, and also because HER2/HER3 expression is reported in ~70% of the human HCC tissue specimens (28, 29).

In conclusion, the results of this experiment support the possibility that inhibition of RXRα phosphorylation and restoration of its physiologic function may be a potentially effective and critical strategy for HCC chemoprevention/chemotherapy. A previous study found that combining 9cRA with vitamin K₂, which targets Ras/MAPK, synergistically inhibits the growth of HCC cells by inhibiting the phosphorylation of RXRα (30). We should also mention the results of a recent phase III, randomized, placebo-controlled trial in HCC patients using sorafenib, which is a multikinase inhibitor with activity against several RTKs and the Raf/MEK/ERK cascade, thus inhibiting the growth of HCC cells (31). In a trial, sorafenib demonstrated an improved overall survival benefit in patients with advanced HCC (32). Thus, the phosphorylation of RXRα might be a potentially useful target for the prevention and/or treatment of HCC, and the combination of retinoids plus a specific Ras/MAPK and/or RTK inhibitor may therefore be a promising strategy for this purpose.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References

Synergistic Growth Inhibition by 9-cis-Retinoic Acid Plus Trastuzumab in Human Hepatocellular Carcinoma Cells

Hideharu Tatebe, Masahito Shimizu, Yohei Shirakami, et al.