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Induction of Apoptosis in a Human Breast Cancer Cell Overexpressing ErbB-2 Receptor by α-Tocopheryloxybutyric Acid

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ABSTRACT—The overexpression of ErbB-2 receptor relates to malignant transformation of breast cancer. The present study was carried out to establish the usefulness of α-tocopheryloxybutyric acid (TE) as a chemotherapeutic agent for human breast cancer. TE caused induction of apoptosis in MDA-MB-453 cells overexpressing the ErbB-2 receptor. TE reduced levels of activated ErbB-2 receptor and Akt. In contrast, TE induced the activation of p38, and SB203580, a specific inhibitor for p38, attenuated the TE-induced apoptosis. These data indicate that simultaneous occurrences of Akt inhibition and p38 activation by TE result in the cell death.

Keywords: α-Tocopheryloxybutyric acid, Apoptosis, Breast cancer cell

Overexpression of ErbB-2 receptor is seen in approximately 30% of human breast cancers, and the overexpression of the receptor is a negative prognostic factor following tumor resection and may be associated with increased resistance to cancer chemotherapy (1). A key mechanism by which the ErbB-2 receptor overexpression stimulates tumor cell growth and renders cells chemoresistant are as follows: survival signal transduction through the ErbB-2 receptor involves the phosphatidylinositol-3 kinase/Akt signaling pathway, and activated Akt is considered the focal point of a survival pathway known to protect cells from apoptosis by several stimuli (2). In a recent report, antisense oligonucleotide-dependent down-regulation of the ErbB-2 receptor in human breast cancer cells results in apoptotic cell death (3). Collectively, these reports encouraged us to develop a new therapeutic agent targeting the overexpressed ErbB-2 receptor in human breast cancer.

Previous in vitro studies using α-tocopheryl succinate (TS) have shown that the antioxidative effect of α-tocopherol is not required for growth inhibition and apoptosis of several tumor cell lines including human breast cancer cells (4). In order to extrapolate this in vitro effect into an in vivo effect, we synthesized one α-tocopherol derivative, α-tocopheryloxybutyric acid (TE), which has no antioxidative effect in vivo (5). Since the ether bond in TE can not be hydrolyzed in vivo, this compound does not show any antioxidative effect (6). In fact, we demonstrated that TE was stable in vivo and inhibited cell proliferation during the carcinogenic process of lung tumorigenesis in mice (5, 7). In that study, we have suggested that TE stabilizes plasma membrane physicochemically and inhibits growth factor receptor-dependent mitogenic signaling, leading to suppression of the cell proliferation (5). Additionally, we found that TE suppressed the activation of epidermal growth factor receptor in a human lung cancer cell line through the stabilizing effect of TE on the plasma membrane (unpublished data). Overall, the stabilizing effect of TE may contribute to the suppression of ErbB-2 receptor activation in human breast cancer cells. In this context, the present study was carried out to clarify this possibility using a human breast cancer cell line, MDA-MB-453 overexpressing the ErbB-2 receptor.

The human breast cancer cell line MDA-MB-453 was provided by Riken Cell Bank (Saitama). The cell was routinely growth in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Tokyo) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. For

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experiments, exponentially growing cells were used. Cells were plated on culture plates and cultured for 24 h to permit them to adhere. After attachment, the medium was changed and cells were cultured for 72 h in DMEM supplemented with 5% FCS containing TS or TE, and each assay was performed. SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) (Calbiochem, San Diego, CA, USA) was pretreated for 6 h before the start of TE treatment.

The cytotoxicity of TE was examined by a WST-1 assay kit (Quick Cell Proliferation Assay Kit; MBL, Nagoya) according to the manufacturer's instructions. In order to estimate apoptosis quantitatively, a sandwich enzyme immunoassay was conducted by using "Cellular DNA fragmentation ELISA" (Roche Diagonistics, Tokyo), according to the manufacturer's instructions, and caspase 3 activity was determined by using a commercial kit (Caspase-3 /CPP32 Colorimetric Assay Kit; Biovision, Moutain View, CA, USA), according to the manufacturer's instructions.

The activations of ErbB-2 receptor, Akt, extracellular signal-regulated kinase (Erk) and p38 were estimated by immunoblot analysis using each antibody against the phosphorylated form of the proteins. Briefly, The cells were lysed in 1 ml of ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerol phosphate, 0.1 mM sodium vanadate, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 mM dithiothreitol fluoride hydrochloride, 1 mM dithiothreitol). The lysates were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and subjected to immunoblotting with anti-phosphorylated ErbB-2 receptor, anti-ErbB-2 receptor (UBI, Lake Placid, NY, USA), anti-phosphorylated Akt, anti-Akt, anti-phosphorylated Erk, anti-Erk, anti-phosphorylated p38 and anti-p38 antibodies (NBL, Beverly, MA, USA). Detection was accomplished using the ECL system (Amersham, Piscataway, NJ, USA) and a cooled CCD camera-linked Cool Saver system (Atto, Tokyo). Molecular sizing was estimated using Rainbow Molecular Weight Marker (Amersham). Protein concentrations were determined by DC Protein Assay kit (Bio-rad, Tokyo).

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple-range test. A P value of 0.05 or less was considered significant.

First, in order to estimate the cytotoxic potential of TE on MDA-MB-453 cells, we compared the potential of TE with that of TS, which was known as a representative agent having a cytotoxic effect on human breast cancer cells (8). TE had almost the same cytotoxicity as TS, and the cytotoxicity reached the maximum level at the treatment condition of 40 mM for 72 h (Fig. 1A). Thus, we selected this treatment condition in this study. Secondly, we compared the apoptosis-inducing effect on the breast cancer cells between TE and TS, using DNA fragmentation and caspase 3 activity as markers of apoptosis. As shown in Fig. 1B, DNA fragmentation and caspase 3 activity in TE-treated cells showed increases with statistical significances compared with those in the control, and the elevations were similar with those in TS-treated cells. Morphologically, we observed characteristics of apoptosis such as round up in the cells (data not shown). These results suggest that the apoptotic effect of TE on MDA-MB-453 cells is similar to that of TS.

Next, we tried to determine the change of survival and/or apoptotic signals contributing to TE-induced apoptosis in MDA-MB-453 cells. Serum-stimulated activations of ErbB-2 receptor and Akt as survival signals were diminished by TE and TS, and the receptor-linked activation of Erk was also abolished by the treatment. In contrast, the activation of p38 was induced by TE and TS (Fig. 1C). Finally, in order to confirm the contribution of the p38 activation to the TE-induced apoptosis, we estimated the effect of a specific inhibitor against p38, SB203580, on the apoptosis and cytotoxicity. As shown in Fig. 2: A and B, the TE-induced DNA fragmentation and elevation of caspase 3 activity were significantly suppressed by SB203580. Furthermore, about 70% of TE-induced cytotoxicity in MDA-MB-453 cells was prevented by SB203580 (Fig. 2C). Taken together with all of our present data, it seems that TE exerts negative growth control in MDA-MB-453 cells due to the inhibition of ErbB-2 receptor survival signals and the activation of p38 apoptotic signals.

TS has been well-characterized with respct to its chemotherapeutic and chemopreventive potential (8). Especially, TS is a potent suppressor of cell growth and inducer of apoptosis in human breast cancer cells, which form solid tumors (9). However, cleavage of the ester bond in TS by an esterase prior to accumulation of the intact TS in tumors would make it ineffective for inhibiting in vivo tumor growth, because α-tocopherol has no effect on cell growth of human breast cancer cells (10). In order to overcome this disadvantage of TS in vivo, we synthesized TE, the ether derivative of TS, which was nonhydrolyzable by esterase, and we demonstrated that the derivative actually inhibited cell proliferation during the chemical carcinogenic process in mice (5). In this study, we found that TE was a potent inducer of apoptosis in MDA-MB-453 cells, like TS. As mentioned above, the overexpression of ErbB-2 receptor in human breast cancer cells is a negative prognostic factor and may be related to increased resistance to cancer chemotherapy (11). Thus, if this in vitro apoptotic inducing effect of TE can be extrapolated to an in vivo effect, TE may be a promising new chemotherapeutic agent against regressive human breast cancers overexpressing the ErbB-2 receptor.

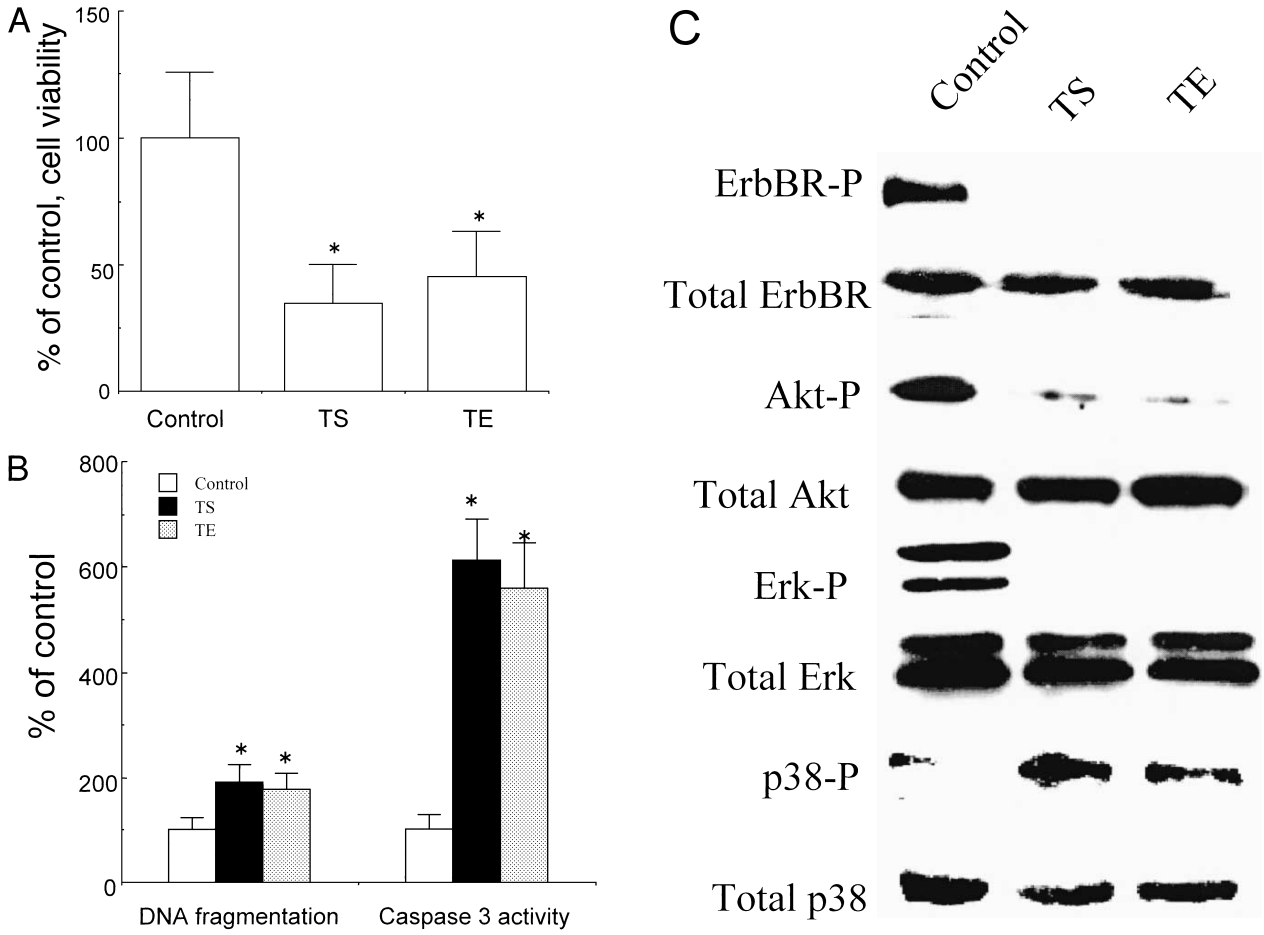

Fig. 1. Effects of TE on cell viability, apoptotic markers, and survival and apoptotic signals in MDA-MB-453 cells. A: Cell viability was determined by WST-1 reagent. Cell culture was carried out in culture medium containing 40 μM of TE or TS. Each column represents the mean of 4 samples and vertical lines indicate S.E.M. Each value is expressed as a percentage of the control. *Significant difference from the control (P<0.05). This result is a representative one of two independent experiments. B: DNA fragmentation and caspase 3 activity were determined by the methods described in Materials and Methods. Cell culture was carried out in culture medium containing 40 μM of TE or TS. Each column represents the mean of 4 samples and vertical lines indicate S.E.M. Each value is expressed as a percentage of the control. *Significant difference from the control. This result is a representative one of two independent experiments. C: The activation of each signal molecule was estimated from the level of each phosphorylated form by immunoblot analysis, as described in Materials and Methods. ErbBR-P, phosphorylated form of ErbB-2 receptor; Akt-P, phosphorylated form of Akt; Erk-P, phosphorylated form of Erk; p38-P, phosphorylated form of p38. Cell culture was carried out in culture medium containing 40 μM of TE or TS. This result is a representative one of two independent experiments.

The balance between cell survival and cell death is a complex issue, and there is considerable effort to understand how tumor cells regulate the decision between these critical pathways (12). A number of clinically useful anticancer agents have been shown to induce apoptosis in tumor cells, and this apoptotic process is believed to be a major component of the therapeutic mechanism (13). In human breast cancer having the overexpression of ErbB-2 receptor, the receptor is considered to be an important target in the treatment of the cancer (3). In fact, clinical validation for employing the ErbB-2 receptor as the target in the cancer treatment has been achieved recently through specific antibody therapies directed toward the receptor (14). On the other hand, a recent report have shown that CI-1033, a potent and specific irreversible inhibitor of the ErbB receptor family can strongly inhibit the ErbB-2-Akt survival signal pathway but not induce apoptosis in the human breast cancer cell lines involving MDA-MB-453 cells (15). Also, this report has demonstrated that, in addition to the inhibition of the receptor activation by CI-1033, other chemotherapeutic agent-induced activation of p38 is required for the cells to commit to apoptosis. From this report, it is considered that the inhibition of Akt and activation of p38 act in concert to promote apoptosis in the

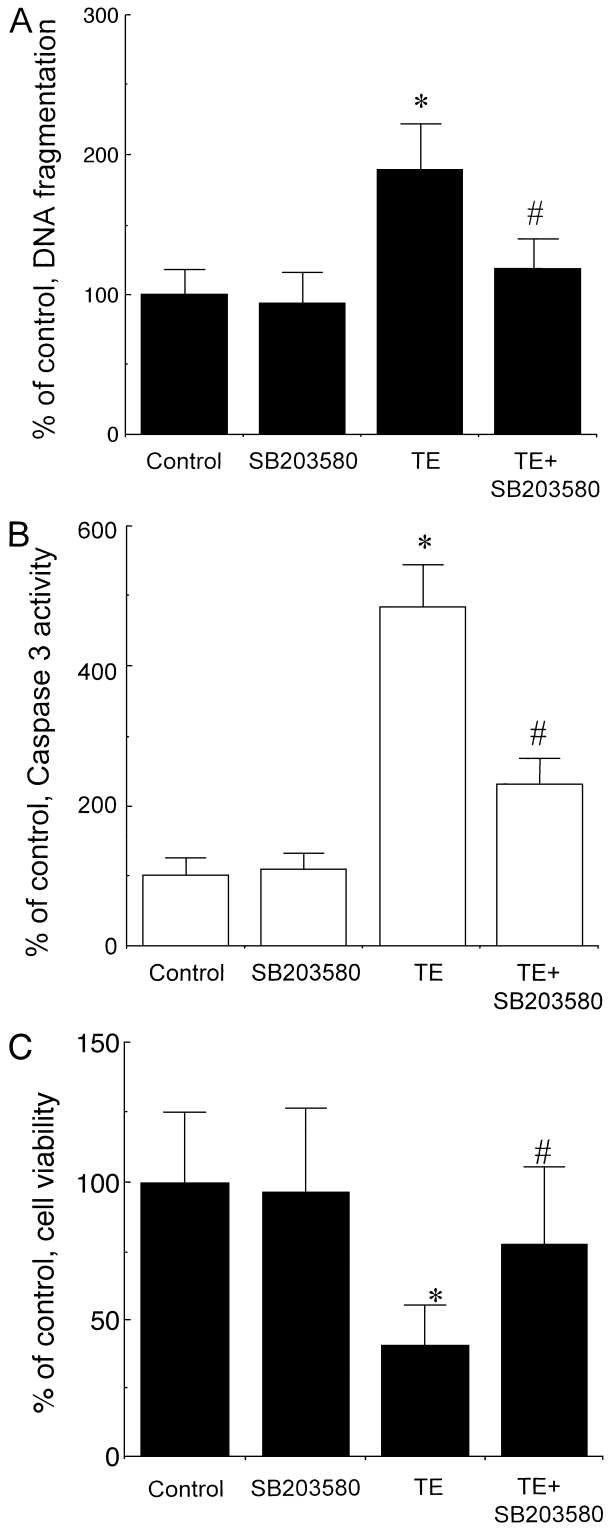

Fig. 2. Effect of SB203580 treatment on TE-induced apoptosis and cytotoxicity in MDA-MB-453 cells. A, B and C: Cell culture was carried out in culture medium containing 20 μM SB203580 and 40 μM of TE. Each column represents the mean of 4 samples and vertical lines indicate S.E.M. Each value is expressed as a percentage of the control. *Significant difference from the control (P<0.05) and $^{\#}$significant difference from the TE-treated cells (P<0.05). This result is a representative one of two independent experiments.

breast cancer cells under the inactivation of the ErbB-2 receptor. Collectively, in order to develop a more potent agent for the treatment of the breast cancers, it is promising for a single agent to have both the ability to inhibit the ErbB-2-Akt survival signals and the ability to activate p38 apoptotic signals. From these criteria, TE is one of the most promising agents for the treatment of the breast cancer cells because it potentially induces apoptosis in the cancer cells through the inhibition of the ErbB-2-Akt survival signals and activation of p38 apoptotic signals, in addition to its stability in vivo as mentioned above.

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