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Selective cytotoxicity of ascochlorin in ER-negative human breast cancer cell lines

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Abstract

While agents targeting estrogen receptors are most effective in adjuvant therapy for human breast cancers expressing estrogen receptors after surgery, breast cancers lacking estrogen receptor are clinically serious, because they are highly malignant and exhibit resistance to the usual anti-cancer drugs, including estrogen receptor-antagonists and DNA breaking agents. Here, we found that MX-1, a human breast cancer cell line lacking estrogen receptors, exhibited higher AP-1 activity and expressed higher levels of c-Jun, c-Fos, and Fra-1 when compared with conventional estrogen receptor-positive human breast cancer cell lines. The prenylphenol antibiotic ascochlorin suppressed the AP-1 activity of MX-1 cells, and selectively killed MX-1 cells, partly due to induction of apoptosis. Our results suggest that AP-1 is an effective clinical target molecule for the treatment of estrogen receptor-negative human breast cancer.

Keywords: Ascochlorin; AP-1; c-Jun; c-Fos; Fra-1; Breast cancer; ER; Apoptosis; Cytochrome *c*; Caspase

Although systemic treatments for breast cancer including the combination of surgery, hormone therapy, chemotherapy, and irradiation are commonly believed to be effective in prolonging patient survival, despite the different genomic backgrounds of each breast cancer, the most important factors for prognosis are estrogen receptor (ER) expression and HER-2 gene expression [1,2]. It is now particularly clear that patients with ER-positive tumors benefit substantially from hormone therapy [3]. For patients with ER-negative tumors, on the other hand, no effective therapy regimens have been established and prognosis is generally poor. It is therefore important to find new agents that are selectively effective against ER-negative breast cancer cells.

Ascochlorin is a prenylphenol antibiotic, originally isolated as an anti-virus agent produced by an incomplete fungus, *Ascochyta visiae* [4,5]. Ascochlorin and its derivatives exhibit a large variety of physiological activities including hypolipidemic activity [6,7], suppression of hypertension [8], amelioration of type I and II diabetes [9,10], immunomodulation [11,12], and anti-tumor activity [12,13]. Ascochlorin and ascofuranone, one of its derivatives, inhibit oxidative phosphorylation by inhibiting ubiquinone-dependent electron transport in isolated mitochondria [14–16], and it is suggested that the anti-viral activity of ascochlorin and the macrophage activation by ascofuranone are caused by this inhibitory activity on mitochondria respiration

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[14,15,17]. They also modulate activity of nuclear hormone receptors, and ascochlorin activates transcription of human ER [18,19], thus suggesting that mechanisms other than those involving the respiratory chain contribute to their physiological activities. We recently found that ascochlorin selectively suppressed the AP-1 activity of human renal carcinoma cells, and its downstream targets such as matrix metalloproteinase-9 promoter (Chang et al., unpublished results).

AP-1 is a family of homodimeric or heterodimeric transcription factors composed of basic region-leucine zipper proteins that belong to the Jun, Fos, and Jun dimerization partners and closely related transcription activating partners [20,21]. AP-1 has been implicated in transcriptional regulation of a wide range of genes participating in cell survival, proliferation, oncogenesis, and apoptosis [21,22].

In this study, we found that AP-1 activity and expression of components of AP-1 significantly increased in ER-negative breast cancer cell lines, and that inhibition of AP-1 activity by ascochlorin selectively reduced their survival. Our results suggest that AP-1 is an effective target for chemotherapy in ER-negative breast cancers.

Materials and methods

Cell culture. Human breast cancer cell lines, MX-1, MDA231, and ZR-75-1, were purchased from American Type Culture Collection (MD, USA) and KPL-1, KPL-4 were kindly provided by Dr. J. Kurebayashi (Kawasaki Medical College, Okayama, Japan) [23,24]. MCF-7 was purchased from Human Cell Science (Osaka, Japan). The human osteosarcoma cell line U2OS was obtained from Nicholas H. Heintz (University of Vermont, Burlington, VT, USA). All cells were cultured and maintained in DMEM supplemented with 5% fetal bovine serum.

Plasmids and antibodies. A luciferase reporter plasmid for AP-1 (pTRE-luc), which contains the AP-1-binding region of the collagenase promoter, was a kind gift from Yvonne M.W. Janssen-Heininger (University of Vermont, Burlington, VT, USA). An expression plasmid for β-galactosidase (pCMV-β-gal) was provided by Nicholas H. Heintz. Antibodies for c-Jun were purchased from Pharmingen (Transduction Laboratories, Lexington, KY, USA), those for ERα, c-Fos, Fra-1, JunB, JunD, caspase-3, and cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), those for poly(ADP-ribose)polymerase were purchased from Oncogene Science (Boston, MA, USA), and those for β-actin were purchased from Abcam (Cambridgeshire, UK).

Western blotting. Exponentially growing cells (2×10^5) were trypsinized and suspended in 50 μl SDS sample buffer (120 mM Tris, 4% SDS, 20% glycerol, 0.1 mg/ml BPB, and 100 mM dithiothreitol, pH 6.8). For cytochrome *c*, the cytoplasmic fraction prepared as described previously [25] was dissolved in SDS sample buffer. After SDS–PAGE, Western blotting of all samples was performed as described previously [26] using first antibodies and the corresponding second antibodies for whole immunoglobulins from mouse or rabbit (Amersham Biosciences, Buckinghamshire, UK). Protein signals were visualized by fluorescence emission using a commercial kit (Roche Diagnosis, Mannheim, Germany).

Reporter assay. Cells were transfected with the plasmids using Fugene transfection reagents (Roche Diagnosis) according to the manufacturer's instructions. One microgram of luciferase reporter plasmids and 100 ng of pCMV-β-gal per 30-mm diameter dish were used for transfection. Luciferase activity (Promega, Madison, WI, USA) and β-galactosidase activity (Promega) were measured using commercial kits as described previously [18,19].

MTT-dye reduction assay. Cell viability of drug-exposed breast cancer cells was determined by dimethylthiazol diphenyltetrazolium (MTT) dye reduction assay, which is based on the measurement of mitochondrial respiratory function [27,28]. Briefly, breast cancer cells (5×10^4 cells/well) were plated in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark), allowed to adhere for 24 h. After incubation, cells were exposed to various concentrations of ascochlorin for 48 h and were then incubated with MTT dye (Roche Diagnosis, 100 μg/well) for 2 h. The resultant formazan deposits were solubilized with 20 μl of 10% SDS, and the absorbance at 590 nm was measured. All data were calculated and expressed as percent A590 of control cells (without treatment, set at 100%).

Nuclear staining. Cells (2×10^5 cells) cultured on a coverslip were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and stained with 3 μg/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) for 15 min. Nuclear morphology of the stained cells was observed using a fluorescence microscope.

Results and discussion

Expression of AP-1 proteins in human breast cancer cell lines was examined by Western blotting (Fig. 1). MX-1 is an ER-negative human breast cancer cell line, while MCF-7 and ZR75-1 are cell lines that express ERα. MX-1 expressed higher levels of c-Fos, Fra-1, and c-Jun when compared with MCF-7 and ZR75-1. However, amounts of JunB and JunD were not significantly different among the cell lines. Although a human osteosarcoma cell line (U2OS) also expressed c-Jun at comparable levels as MX-1, expression of c-Fos and Fra-1 was as low as that in MCF-7 and ZR75-1. This suggests that the high-level expression of Fos-family proteins is one of the features distinguishing ER-negative and ER-positive breast cancer cell lines.

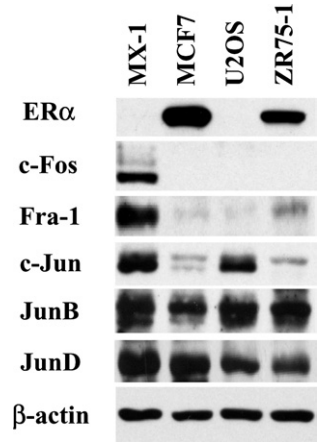

Fig. 1. Selective expression of AP-1 proteins in ER-negative or positive human breast cancer cell lines. Exponentially growing cells (5×10^5 cells) were lysed in 50 μl SDS sample buffer and subjected to Western blotting.