The regulatory effect of tamoxifen on fibronectin expression in estrogen-dependent MCF-7 breast carcinoma cells

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Abstract. We investigated the regulatory effect of tamoxifen (TAM) on fibronectin (FN) expression in estrogen-dependent MCF-7 breast carcinoma cells both in vitro and in vivo. In vitro, MCF-7 cells were cultured with 17β-estradiol (E2) and/or TAM. In the animal experiment in vivo, MCF-7 tumors were grown in ovariectomized athymic mice by implanting a sustained release E2 pellet. The E2 pellets were removed after 3 weeks of E2 treatment. Animals were then divided into four groups: 1) an E2 (0.72 mg/pellet) pellet [E2(+)]; 2) an E2 and a TAM (5 mg/pellet) pellet [E2(+)(TAM)]; 3) no treatment [E2(-)]; and 4) a TAM pellet [E2(-)(TAM)]. Following each treatment for 4 weeks, all animals were sacrificed and tumors were removed. Specimens, cells (in vitro) or tumors (in vivo), were homogenized and assayed for FN by Western blots. In the in vitro experiment, FN expression in MCF-7 cells decreased by incubating with 10^{-9} M E2 and increased with 10^{-6} M TAM. The effect of TAM increasing FN expression was inhibited by incubation accompanied with 10^{-9} M E2 or 1 μg/ml transforming growth factor-β (TGF-β) neutralizing antibody. In the in vivo animal experiment FN expression in the tumors of E2(+)(TAM) mice was lower than that of E2(-) mice. However, TAM increased FN expression in the tumors regardless of E2 pellet. These results suggest that TAM increases FN expression of MCF-7 breast carcinoma cells and that these regulatory effects of TAM on FN expression are partly mediated by TAM-induced TGF-β.

Introduction

Fibronectin (FN) is a large glycoprotein found ubiquitously in the plasma and other body fluids (plasma FN), in extracellular matrices, on the surface of cells, and in basement membranes (cellular FN) (1,2). FN plays a key role in the tissue remodeling and cell migration events that occur during normal embryonic development and adult wound healing (3), and it has been thought to have an important role in both tumor invasion and metastasis (4).

FN has been proven to be produced by various cancer cells including breast cancer (5-7). Takei and his co-workers reported that FN, which is deposited in the stroma surrounding breast cancer cells, analyzed by immunohistochemistry was a prognostic indicator of patients with breast cancer. It was also shown that the relapse-free survival of patients with FN-positive tumors was significantly better than patients with FN-negative tumors (8).

Cellular FN is a major constituent of the cell surface of many cultured cells, and it is either eliminated or reduced in presence on the surface of oncogenically transformed cells (1,2). Several studies have suggested that there is a correlation between the loss of cell surface FN and the ability of a cell to metastasize (9-11).

FN expression is regulated by a variety of growth factors and hormones, such as glucocorticoids, transforming growth factor-β (TGF-β), cAMP, epidermal growth factor, platelet-derived growth factor, vitamin D3, and estrogen (12,13). However, the regulatory mechanism of FN expression of breast carcinoma is not clear. Investigation of the regulatory mechanism is, therefore, expected to lead to the elucidation of suppressive regulation of the metastasis of breast cancer.

Tamoxifen (TAM), a non-steroidal antiestrogen, is a standard treatment medicine for patients with estrogen receptor-positive breast cancer. Five years of TAM has been shown to significantly improve long-term survival of patients with estrogen receptor-positive breast cancer (14). Although the antitumor action of TAM is to competitively block estrogens from binding to the estrogen receptor of breast cancer, the whole process of this antitumor action of TAM remains to be clarified.

TAM reportedly stimulated the production of TGF-β in vitro (15) and in vivo (16), and inhibited that of vascular endothelial growth factor (VEGF) in vitro in breast cancer (17). However, few investigations concerning the effect of TAM on FN expression in breast cancer have been reported. Thus, we were interested in whether or not TAM effected FN expression in estrogen-dependent breast carcinoma cells.
and diet (MF, Oriental Yeast Co., Ltd., Tokyo) were given.

In the present study, we evaluated the regulatory effect of TAM on FN expression in estrogen-dependent MCF-7 breast carcinoma cells in vitro and in vivo.

Materials and methods

Media and chemicals. E2 and TAM were obtained from Sigma Chemical Company (St. Louis, MO). TGF-ß1 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN). E2 (0.72 mg/pellet, 60-day sustained release) and TAM (5 mg/pellet, 60-day sustained release) pellets were obtained from Innovative Research of America (Sarasota, FL). Immobilon™ PVDF (polyvinylidene difluoride) transfer membrane for Western blotting was from Millipore Corp. (Bedford, MA). ECL™ Western blotting detection reagent was from Amersham International (Bedford, MA). The antibody to FN was from Oncogene Research Products (Cambridge, MA). The secondary antibody, horseradish-peroxidase-linked sheep anti-mouse IgG was from Amersham International Plc. (Buckinghamshire, UK). The antibody to FN was from Oncogene Research Products (Cambridge, MA). The secondary antibody, horseradish-peroxidase-linked sheep anti-mouse IgG was from Amersham International. Bio-Rad protein assay dye reagent was from Nippon Bio-Rad (Tokyo). Other reagents were of the highest grade available.

Cell culture. Human breast carcinoma cell lines MCF-7 were generously provided by Dr Robert Dickson (Georgetown University, Washington, DC). The cells were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco BRL) and were grown in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Tumors in nude mice and treatment groups. Athymic mice [BALB/c (nu/nu)] were provided by the Institute of Experimental Animal Research, Gunma University. Sterilized water and diet (MF, Oriental Yeast Co., Ltd., Tokyo) were given.

ad libitum. Room temperature (24°C), humidity (40%), and 12 h of light were automatically controlled. A total of 4x106 cells of MCF-7 were inoculated bilaterally into the thoracic mammary fat pads (1/side) of 6-week-old athymic mice, which were ovariectomized at 5 weeks old. All animals were also implanted subcutaneously with an E2 pellet because ER-positive MCF-7 tumors fail to grow in the absence of estrogen. After 3 weeks of estrogen treatment, tumors had reached an average size of 0.5 cm3. The E2 pellet was removed from the animals and randomized into groups (6 animals in each group); and then received one of the following treatments: i) an E2 pellet [E2(+)]; ii) an E2 and a TAM pellet [E2(+)TAM]; iii) no treatment [E2(-)]; and iv) a TAM pellet [E2(-)TAM]. Tumor measurements were performed using slide calipers. Tumor sizes were obtained using the formula (length x width x length x width x π). The size of each tumor was recorded weekly as a percentage of the initial tumor size at day 0 of treatment, and mean values ± (SE) percentage changes at specific time points were calculated for each group. After 4 weeks, all animals were sacrificed, tumors were removed and cleared of all skin, fat, and obvious necrosis, and snap-frozen in liquid nitrogen. Frozen tumor specimens were stored at -80°C until the experiments.

Cell proliferation assay. Prior to the following assays in vitro, MCF-7 cells were cultured in phenol red-free RPMI-1640 supplemented with 2% charcoal-stripped FCS for at least 3 days. MCF-7 cells were harvested with 0.1% trypsin and 0.05% EDTA in PBS and were plated into 96-well multiplates at 3x104 cells/well. A day later, fresh medium both with and without E2 (10-8 M) and TAM (10-6 M), respectively, were added. Forty-eight hours later, fresh medium containing test substances was replaced. Cells were cultured for DNA assays. Following the incubation for various number of days, wells containing ethanol-fixed cells...
were allowed to dry. The DNA content of each well was measured according to Heinegardner (18).

Sample preparation for grown cells in vitro. MCF-7 cells, as described previously, cultured in phenol red-free RPMI-1640 supplemented with 2% charcoal-stripped FCS for at least 3 days, were harvested with 0.1% trypsin and 0.05% EDTA in PBS and were plated into culture dishes. A day later, fresh medium with or without E2 (10⁻⁹ M) and TAM (10⁻⁶ M), respectively, were added. Forty-eight hours later, fresh medium containing test substances was replaced. Cells were cultured for analysis of cell surface FN. Following incubation for 96 h, cells were collected by scraping. The extraction buffer for analysis of FN contained 10 mM Tris-HCl, pH 7.5; 0.25 M sucrose; 5 mM EDTA; 50 mM NaCl; 30 mM sodium pyrophosphate; 50 mM sodium fluoride; 100 mM sodium orthovanadate; 1 mg/ml pepstatin A; and 2 mg/ml leupeptin. PMSF (phenylmethylsulfonyl fluoride) (1 mM) was added just before use. Cells were lysed by the extraction buffer mixed with 0.2% Triton X-100 and homogenized using an ultrasonic cell disruptor Microson XL-2005 (Heat Systems, Farmingdale, NY) on ice. All debris and nuclei were removed by centrifugation at 900 x g for 10 min (4°C). All the supernatants were centrifuged at 105,000 x g for 60 min (4°C). The pellet thus obtained was resuspended in the extraction buffer, mixed with 0.2% Triton X-100 and used as the solubilized membrane fraction for analysis of FN.

Western blot analysis. Western blotting was performed according to the modified Laemmli method described in detail previously (19,20). Proteins (50 μg for FN) were separated with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 5% (w/v) running gel containing acrylamide and N,N'-methylene-bis-acrylamide in the ratio of 37:1 in the presence of 0.1% (w/v) SDS, and then transferred to Immobilon™ PVDF membranes. The PVDF membranes were incubated with 5% skim milk in Tris-buffered saline (TBS). After washing 3 times in 0.05% Tween-20-TBS (TBST), the membrane was incubated with 1 mg/3 ml antibody to FN in the TBST for 2 h at room temperature. After rinsing 3 times in TBST, a secondary antibody solution consisting of 1 mg/3 ml horseradish-peroxidase-linked sheep anti-mouse Ig and TBST was added, and incubated for 1 h at room temperature. Proteins were detected with the ECL™ Western blot detection reagent.

Protein assays. Protein concentrations were determined with bovine serum albumin as the reference standard using the Bio-Rad protein assay dye reagent (21).

Statistical analysis. Values are expressed as the mean ± the standard error of the mean (SEM). A statistical difference was analyzed between two groups using the Student’s t-test or among more than three groups using the Fisher’s and the Scheffe’s test with an analysis of variance (ANOVA). StatView (SAS Institute, Cary, NC) was used for all statistical analyses and p-values of <0.05 were considered to be significant.

Results

Effects of E₂ and TAM on cell growth in MCF-7 cells. Fig. 1 shows the effects of E₂ and TAM on cell growth in MCF-7 cells in vitro. When MCF-7 cells were incubated with E₂ at a concentration of 10⁻⁹ M for 4 days, cell growth was significantly (p<0.01 vs. control) stimulated. By contrast, after the cells were incubated with TAM at a concentration of 10⁻⁶ M for 4 days, cell growth was reduced to ~50% of the control level (p<0.01 vs. control). Furthermore, when MCF-7 cells were incubated with TAM (10⁻⁶ M) together with E₂ (10⁻⁹ M), the stimulating effect of E₂ on cell growth was inhibited to the approximate control level.

Growth of MCF-7 tumors in vivo. Prior to the randomization, E₂-stimulated tumor growth was observed in all mice. Fig. 2
Fig. 3 shows the effects of E2 and TAM on FN expression in MCF-7 tumors in the in vivo experiment. Sample preparation for grown tumors in the in vivo experiment is described in Materials and methods. FN expression in the tumors of E2(+)-mice was lower than that of E2(-)-mice. TAM increased FN expression in the tumors regardless of E2 pellet.

shows the effects of E2 and TAM on tumor growth of MCF-7 tumor implanted in athymic mice. Tumor growth continued in the E2(+)-group. The tumor size was reduced significantly (p<0.01) in the E2(-)-group, compared with the E2(+)-group. TAM significantly inhibited MCF-7 tumor growth in the high estrogen environment [E2(+)-vs. E2(+)-TAM, p<0.01]. Tumor growth was blocked and nearly ceased after treatment in the E2(-)-TAM group. However, TAM did not induce further reduction of the tumor size in the low estrogen environment [E2(-)-vs. E2(-)-TAM, NS].

Levels of FN expression in MCF-7 cells in vitro. Specimens, cells (in vitro) or tumors (in vivo), were homogenized and assayed for FN by Western blots. The antibody to FN reacted with all samples at a position of the protein weight ~220 kDa. FN expression was, however, inhibited by incubation with 10^-9 M E2 and increased with 10^6 M TAM. The effect of TAM increasing FN expression was inhibited by incubation with 10^6 M E2 (Fig. 3A).

FN expression is thought to be regulated by a variety of growth factors such as TGF-ß, and therefore, we examined whether or not the regulatory effects of TAM on FN expression were mediated by TGF-ß. FN expression upregulated by incubation with TAM was decreased by incubation with 1 μg/ml of TGF-ß1 neutralizing antibody (Fig. 3B). These results suggested that TAM increased FN expression of MCF-7 breast carcinoma cells and that these regulatory effects of TAM on FN expression were at least partly mediated by TGF-ß.

Levels of FN expression in MCF-7 tumors in vivo. Fig. 4 shows the effects of E2 and TAM on FN expression in MCF-7 tumors in the in vivo experiment. FN expression in the tumors of E2(+)-mice was lower than that of E2(-)-mice. However, TAM increased FN expression in the tumors regardless of the E2 pellet. These results suggested that TAM increased FN expression of MCF-7 breast carcinoma cells, also in vivo, and that the regulatory effect of TAM on FN in vivo differed somewhat from that of in vitro.

Discussion

Several studies suggest that FN is related to tumor invasion and metastasis (4,7,9-11,22,23). Takei and his co-workers reported that the stromal FN expression analyzed was a prognostic indicator of patients with breast cancer (8) and that microvessel count, which correlated with lymph node metastasis, TNM stage, recurrence, and mortality, was relatively low in stromal FN-positive tumors (24). Thus, from the point of view of invasion and metastasis, it is important to investigate the inducers on FN expression of carcinoma.

In the present study, E2 stimulated growth and inhibited cellular FN expression of estrogen-dependent MCF-7 carcinoma, both in vitro and in vivo. Although E2 is the most potent growth stimulator for ER-positive breast carcinomas; its effects on other carcinoma cells are various. In addition, its effect on FN expression of carcinoma cells remains to be clarified. For example, Landström and his colleagues (13) reported that the growth of rat prostatic adenocarcinoma (Dunning R3327) was inhibited by castration and estrogen treatment, and that FN of both plasma and cellular types increased. The effect of E2 on FN expression is contrary to its effect on tumor growth of certain carcinomas. Inducers that stimulate FN expression of carcinoma are likely to suppress the progression of carcinoma.

We used 60-day sustained release pellets of E2 and TAM to treat tumor-bearing athymic mice. The E2 level was targeted to be within the range normally observed in premenopausal patients during TAM therapy (300-400 pg/ml). The TAM level we selected was 3-4 ng/ml, which was slightly lower than clinical dosage.

Cellular FN expression in MCF-7 cells increased by TAM, both in vitro and in vivo. The stimulating effect of TAM on FN expression was, however, inhibited by incubation with E2 in vitro, by contrast, it was observed regardless of E2 in vivo. Thus, the in vivo regulation of FN by TAM differs from that in vitro. FN expression is regulated by a variety of growth factors and hormones in vivo; thus, the effects of TAM might be influenced by such mediators. Based on the in vivo analyses, TAM might increase FN expression either in premenopausal or postmenopausal patients.

The correlation between growth of MCF-7 cells or tumors and FN expression is notable. In vitro, when MCF-7 cells were incubated with TAM (10^6 M) together with E2 (10^-8 M), cell growth approximated that of control level; however, FN expression under TAM with E2 treatment decreased below that of the control level. In vivo, to the contrary, tumor growth of the E2(-)-TAM group approximated to the level of the E2(+) group, but FN expression in the tumors of the E2(-)-TAM group increased more than that of the E2(+) group. From our results, it was suggested that growth of carcinoma and FN expression are not always inverse.

TGF-ß is thought to stimulate FN expression (12). Knabbe and co-authors demonstrated that MCF-7 carcinoma cells secreted TGF-ß and that antiestrogens increased the secretion by MCF-7 cells (15). We examined whether or not the regulatory effects of TAM on FN expression were regulated by TGF-ß. TGF-ß1 neutralizing antibody has been found to be capable of neutralizing a number of physiological effects produced by TGF-ß. By incubation with TGF-ß neutralizing
antibody, FN expression upregulated by TAM decreased in vitro. The results suggest that the stimulating effect of TAM on FN expression of MCF-7 breast carcinoma cells is at least partly mediated by TAM-induced TGF-β.

We conclude that TAM has a stimulating effect on FN expression in MCF-7 breast carcinoma cells both in vitro and in vivo, and that the stimulating effect of TAM on FN expression might be at least partly mediated by TAM-induced TGF-β. These results suggest that TAM has suppressive effects on invasion or metastasis potential, from the point of view of FN expression, as well as cell proliferation of breast cancer.

References