Tamoxifen Restores the E-Cadherin Function in Human Breast Cancer MCF-7/6 Cells and Suppresses Their Invasive Phenotype

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Abstract

Tamoxifen is an antiestrogen used in adjuvant therapy of breast carcinoma and could potentially prevent the development of mammary cancer. While it is widely clinically used, its exact mechanisms of action are not yet fully elucidated. MCF-7/6 cells are estrogen receptor-positive invasive human breast cancer cells with a functionally inactive cell surface E-cadherin. In this study, we report that tamoxifen, and to a lesser extent its metabolites 4-OH-tamoxifen and N-desmethyl-tamoxifen, restore the function of E-cadherin in MCF-7/6 cells. In an aggregation assay, 10⁻⁹ M tamoxifen significantly increases the aggregation of MCF-7/6 cells. This effect is abrogated by a monoclonal antibody against E-cadherin (HECD-1), is fast (within 30 min), and does not require de novo protein synthesis. Tamoxifen was also found to inhibit the invasion of MCF-7/6 cells in organ culture. Our data is the first demonstration that tamoxifen can activate the function of an invasion suppressor molecule and suggest that the restoration of E-cadherin function may contribute to the therapeutic benefit of tamoxifen in breast cancer patients.

Introduction

Tamoxifen is certainly the nonsteroidal antiestrogen the more prescribed in adjuvant therapy of human breast cancer. Recent studies indicate that it could reduce the incidence of breast cancer in high risk patients. Such observation has led to large scale international randomized trials to evaluate the potential of tamoxifen to prevent this deadly disease. Tamoxifen has been found to act on several targets implicated in breast cancer progression. It inhibits estrogen binding to its receptor (1), increases the secretion of transforming growth factor β (2), reduces the secretion of transforming growth factor α (3), and reduces cell-matrix adhesion (4). Invasive tumors are indeed ecosystems in which neoplastic cells interact with host cells under the influence of matrix molecules and soluble factors (5). The elements that enter into the constitution of such an ecosystem are potential targets for anti-invasive agents. The cell-cell adhesion molecule E-cadherin is a powerful invasion suppressor molecule, and invasiveness has been correlated with down-regulation or functional inactivity of this molecule at the cell surface (6). In this study, we tested the possibility that tamoxifen could act on the E-cadherin cell-cell adhesion and invasion suppressor functions in human mammary carcinoma cells. To test this hypothesis, human MCF-7/6 mammary adenocarcinoma cells were used. They are invasive in vitro (7, 8) and in vivo (9) and do express a functionally inactive E-cadherin at their cell surface (10). The cell-cell adhesion function of E-cadherin was studied in a fast aggregation assay, while its invasion suppressor function was evaluated in confronting cultures of the MCF-7/6 cells with fragments of embryonic chick heart. In these models, we decided to investigate the effects of tamoxifen and its metabolites (4-OH-tamoxifen and N-desmethyl-tamoxifen) in vitro.

Materials and Methods

Cells. Human MCF-7/6 mammary adenocarcinoma cells (11) were obtained from Dr. Henri Rochefort (Unité d’Endocrinologie Cellulaire et Moléculaire, Montpellier, France). They were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 50:50 (Flow, Irvine, Scotland) supplemented with 0.05% glutamine (w/v), 250 international units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. They were Mycoplasma-free after treatment with Mycoplasma removal agent (Dainippon Pharmaceutical Corporation, Osaka, Japan). MCF-7/6 cells express both E-cadherin (10) and estrogen receptors (12).

Chemicals and Antibodies. Tamoxifen, 4-OH-tamoxifen, and N-desmethyl-tamoxifen were kindly provided by Besin-Iscovesco (Paris, France). Stock solutions of 5 X 10⁻⁴ M in ethanol were prepared for further dilution in culture medium and kept in the dark. Cycloheximide was from Sigma Chemical Co., St. Louis, MO. The monoclonal anti-E-cadherin HECD-1 was a gift from Dr. Y. Shimoyama (Pathology Division, National Cancer Center Research Institute, Tokyo, Japan), and αR3, a monoclonal antibody against the insulin-like growth factor receptor, was obtained from Oncogene Sciences, Uniondale, NY.

Assay for Fast Cell Aggregation. This assay was based on first preparing a cell suspension under E-cadherin saving conditions and then measuring cell aggregation in a Ca²⁺-containing as compared to a Ca²⁺-free medium (10). The number of particles was measured with a Coulter counter 2M (Coulter Counter Electronics, Luton, United Kingdom) at the start of the incubation (N₀) and after 30 min (Nₙₐ₃) in suspension. Cells were incubated with various concentrations of tamoxifen or its metabolites. In control experiments, ethanol alone was added so the final concentration was identical to the one in the experimental conditions. All experiments were done at least twice.

Samples of MCF-7/6 cells taken at the start and after 30 min of incubation were fixed and stained immunocytochemically with HECD-1 as a primary antibody and with a fluorescein isothiocyanate-labeled anti-mouse antiserum as a secondary antibody, for localization of E-cadherin with the fluorescence microscope.

Indirect Immunofluorescence Coupled to Flow Cytometry. MCF-7/6 cells (5 x 10⁵), detached from stock cultures under E-cadherin saving conditions, were incubated with HECD-1 and a rabbit anti-mouse antiserum conjugated with fluorescein isothiocyanate (10). Fluorescence intensity was measured with a FACScan III (Becton Dickinson, Mountain View, CA).

Assay for Invasion. The assay for invasion was based on the confrontation in vitro between cell aggregates and chick heart fragments in organ culture (13). Briefly, 9-day-old embryonic chick heart fragments were precultured and selected for a diameter of 0.4 mm. These PHF³ were confronted with aggregates of MCF-7/6 cells with a diameter of about 0.2 mm. After an overnight incubation on top of semi-solid agar, the confronting pairs were cultured in suspension for another 7 days. After fixation in Bouin-Holland’s solution, the cultures were embedded in paraffin, serially sectioned, and stained with ³ The abbreviations used are: PHF, precultured heart fragments; IGF-I, insulin-like growth factor I.
hematoxylin and eosin. In alternating sections, the PHF was stained immunohistochemically with a polyclonal antiserum against chick heart. The interaction between MCF-7/6 cells and PHF was evaluated histologically.

The number of cultures examined for each group was between five and seven. Cultures were treated with tamoxifen at concentrations ranging from $10^{-11}$ to $10^{-6}$ M for 7 days. The tamoxifen metabolites were tested at $10^{-6}$ M only.

**Results**

**Fast Cell Aggregation.** In the presence of Ca$^{2+}$, MCF-7/6 cells showed poor spontaneous cell aggregability, and treatment with the solvent ethanol (2%) showed no effect in this assay. Tamoxifen, tested at concentrations ranging from $10^{-11}$ M to $10^{-3}$ M, increased MCF-7/6 aggregation at $10^{-6}$ M and $10^{-5}$ M but not at lower concentrations (Fig. 1). These increases were statistically significant with Student’s t test ($P < 0.005$). The metabolites 4-OH-tamoxifen and N-desmethyl-tamoxifen were tested at the same molar concentrations as tamoxifen, and they also increased MCF-7/6 cell aggregation at $10^{-6}$ M and $10^{-5}$ M ($P < 0.01$). The metabolites, however, were less potent than tamoxifen itself (Fig. 1). To exclude cytotoxicity effects during the 30-min treatments, a sample of each suspension was explanted on a tissue culture plastic substrate and incubated in drug-free medium for 24 h. In all cases, outgrowth on the substrate was noted without differences between cells treated with tamoxifen and the solvent.

The increase of fast cell aggregation by tamoxifen was E-cadherin dependent. In the absence of Ca$^{2+}$, no increase of MCF-7/6 cell aggregation could be observed by tamoxifen (Fig. 2). This finding points to the implication of a Ca$^{2+}$-dependent cell-cell adhesion molecule in the tamoxifen effect. HECD-1, a monoclonal antibody that blocks the function of E-cadherin, was able to inhibit the tamoxifen effect completely. The latter experiments were done in the presence of Ca$^{2+}$ and at an antibody dilution of 1:20.

Indirect immunofluorescence coupled to flow cytometry showed that tamoxifen did not alter the expression of E-cadherin at the surface of MCF-7/6 cells (Fig. 3).

The tamoxifen effect appears to be independent from de novo protein synthesis, since 10 $\mu$g/ml cycloheximide did not affect the fast aggregation of MCF-7/6 cells induced by tamoxifen.

IGF-I is able to stimulate E-cadherin-dependent aggregation of MCF-7/6 cells, and its effect can be blocked by aIR3, a monoclonal antibody against the IGF-I receptor. At 15 $\mu$g/ml, this antibody, however, was unable to inhibit the effect of tamoxifen. This finding suggests that the tamoxifen-induced increase of MCF-7/6 cell aggregation does not require external triggering of the IGF-I receptor.

![fig1](image1.png)

**Fig. 1.** Dose-response curve of tamoxifen and its metabolites on fast MCF-7/6 cell aggregation. Aggregation is expressed as $1-N_m/N_0$, where $N_0$ is the initial number of particles in suspension, and $N_m$ is the number after 30 min of treatment.

![fig2](image2.png)

**Fig. 2.** Effects of Ca$^{2+}$, cycloheximide, and monoclonal antibodies on tamoxifen-induced fast aggregation of MCF-7/6 cells. Tamoxifen (TAM) was unable to induce aggregation in the absence of Ca$^{2+}$ and in the presence of a monoclonal antibody against E-cadherin (HECD-1). Cycloheximide (CHM) and a monoclonal antibody against the insulin-like growth factor receptor (aIR3) did not affect the tamoxifen effect. Data, mean; bars, SD.

![fig3](image3.png)

**Fig. 3.** Indirect immunofluorescence of E-cadherin coupled to flow cytometry. MCF-7/6 cells were treated for 30 min with $10^{-6}$ M tamoxifen (C) or with the solvent only (A and B). Indirect immunofluorescence was based on the application (B and C) or the omission (A) of a primary monoclonal antibody against E-cadherin. Tamoxifen did not alter the expression of E-cadherin at the surface of MCF-7/6 cells. Ordinate, number of cells; abscissa, fluorescence intensity.

Although the degree of aggregation was quite different after tamoxifen treatment, a similar E-cadherin immunofluorescence pattern was observed in both tamoxifen- and solvent-treated cells. In both situations, E-cadherin was present at the plasma membrane of aggregating cells, and the immune signal was somewhat more accentuated at the cell-cell contact sites as compared to the free sites.

**Invasion.** Histological analysis of confronting cultures between MCF-7/6 cells and PHF revealed occupation and replacement of the PHF by the cancer cells after 8 days of incubation. The number of
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cultures showing invasion versus the total number of cultures in this group was 7 of 7. Treatment of the confronting cultures with the solvent ethanol did not alter the invasion of the MCF-7/6 cells (7 of 7). When tamoxifen was added to the culture medium at the same concentrations as mentioned for the fast aggregation assay, an antiinvasive effect was noticed only at a concentration as high as $10^{-6}$ M (1 of 7). This effect was clear from sections stained with hematoxylin and eosin but was even more obvious from immunohistochemical stainings against chick heart. The antiinvasive effect of tamoxifen was reversible; when 8-day-treated confrontations were cultured for another 8 days in drug-free medium, invasion resumed (5 of 5). No histological signs of cytotoxicity were observed in tamoxifen-treated cultures.

The metabolites 4-OH-tamoxifen and N-desmethyl-tamoxifen did not inhibit invasion of MCF-7/6 cells in PHF at $10^{-6}$ M (7 of 7 for each metabolite).

Discussion

We have shown that tamoxifen activates E-cadherin at the plasma membrane of MCF-7/6 cells, increases cell aggregation, and inhibits invasion in vitro. Evidence for the invasion suppressor role of E-cadherin stems from experiments in vitro in which invasiveness was switched on and off by respective down- and up-regulation of the expression or function of E-cadherin (14, 15). Histological studies have shown that down-regulation of the E-cadherin immunosignal is found in carcinomas of many organs including human breast (16, 17). The invasive MCF-7/6 cells express a functionally inactive E-cadherin and show poor cell aggregation. Until now, four molecules have been shown to increase MCF-7/6 cell aggregation via E-cadherin activation: IGF-I, retinoic acid (18), the flavonoid tangeretin (19), and tamoxifen. These molecules inhibit the invasion of MCF-7/6 cells into PHF at the effective concentrations of the fast cell aggregation assay (8, 19).

The mechanism behind the increase of cell aggregation induced by tamoxifen is only partly understood. Evidence for the implication of E-cadherin in this effect comes from the findings that it is Ca$^{2+}$ dependent and that it is abrogated by HEC1-1, a monoclonal antibody against human E-cadherin. The tamoxifen effect is fast, being observable within 30 min, and it is not dependent on de novo protein synthesis. Alterations of the distribution of E-cadherin over the plasma membrane appear not to be implicated. As triggering of the IGF-I receptor is a prerequisite for intracellular signal transduction to E-cadherin during treatment with IGF-I we wondered whether tamoxifen might also act via the external domain of this receptor. Blocking experiments with αL3, a monoclonal antibody against the IGF-I receptor, however, made this hypothesis unlikely, although we cannot exclude activation of the cytoplasmic tyrosine kinase. In this context, we can add that tamoxifen modulates the activity of various enzymes such as protein kinase (20), Ca/Mg-ATPase (21), and calmodulin-dependent cAMP phosphodiesterase (22). The rather sharp transition of the effect on aggregation between $10^{-7}$ M and $10^{-6}$ M tamoxifen suggests that a possible compensatory mechanism is saturated at $10^{-6}$ M. The nature of such a mechanism, however, is unknown and is the subject of our current research. Probably a membrane accumulation of tamoxifen, which eventually induces a change in the physical properties (fluidity) of the lipid bilayer, is responsible for this uncommon dose-response curve (23). Implication of the estrogen receptor in the tamoxifen effect is suggested by our observation that two estrogen receptor-negative human breast cell lines (SK-BR-3 and HBL-100) were insensitive to tamoxifen in the fast aggregation assay (data not shown).

Tamoxifen is more potent than its metabolites 4-OH-tamoxifen and N-desmethyl-tamoxifen for increasing MCF-7/6 cell aggregation and inhibiting invasion. Yet, relatively high concentrations (10$^{-6}$ M or higher) are required for these effects. Studies on intratumoral tamoxifen concentrations, however, have shown that 10$^{-6}$ M is usually exceeded in mammary carcinomas (24). We therefore believe that the restoration of the E-cadherin function in human mammary carcinoma cells may contribute to the therapeutic benefit of tamoxifen in breast cancer patients.

References

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