Communications of Neu/c-erbB-2 Receptor and Inhibits Growth of Human Malignant Glioma Cell Lines Following Tamoxifen Treatment

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Abstract: In the present study, effects of tamoxifen, an antiestrogen, on the inhibition of protein tyrosine phosphorylation in neu/c-erbB-2 receptor, DNA synthesis and proliferation were evaluated using the malignant glioma cell lines U25 IMG and T98G which overexpressing neu/c-erbB-2. Pre-treatment of two cell lines with tamoxifen resulted in a dose dependent inhibition of tyrosine phosphorylation as well as DNA synthesis and cell growth in two cell lines correspondingly. The results support the hypothesis that activated protein tyrosine kinase receptors are involved in the proliferation of glioma cells. Tamoxifen may be useful in the treatment of malignant glioma.

Key words: Tamoxifen, neu/c-erbB-2, protein tyrosine phosphorylation, DNA, glioma

INTRODUCTION

The human neu/c-erbB-2 proto-oncogene encodes a 185 kilodalton (kD) transmembrane glycoprotein, neu protein, with intrinsic tyrosine kinase activity that resembles the Epidermal Growth Factor Receptor (EGFR). The neu/c-erbB-2 gene and protein were originally identified in the brain and are thought to play a critical role in neurogenesis. The overexpression of the gene for the neu protein appears to be correlated with aggressiveness of various tumors. Protein Tyrosine Kinase (PTK) receptors that have been implicated in a wide variety of physiological functions and tyrosine kinase are involved in the signal transduction cascades from the growth factor receptor towards the deoxyribonucleic acid (DNA) replication system in the nucleus. An imbalance in the phosphorylation state of signal-transducing proteins is thought to contribute to deregulated cell proliferation. In several types of tumors, increased protein PTK activity was found. In some tumors, PTK activity correlated with histology, lymph node metastasis, or prognosis. Tamoxifen, [(Z)-2[p-(1,2-diphenyl-1-butenyl) phenoxy]-N, N-dimethyl ethylamine citrate (1:1)], a synthetic, nonsteroidal antiestrogen compound, which has been used extensively in the treatment of estrogen receptor (ER)-positive breast cancer. Tamoxifen was reported to have inhibited the growth of some ER-negative cell lines of malignant glioma. It has been reported that tamoxifen inhibits protein kinase C (PKC) by interfering with its activity of the catalytic subunit. Tamoxifen can also penetrate the blood-brain barrier, which suggests a possible role for it in the treatment of malignant glioma. We report here the effects of tamoxifen on the tyrosine phosphorylation of neu protein and its influence on the DNA synthesis and proliferation in two human glioma cell lines.

MATERIALS AND METHODS

Cell culture: The human glioblastoma cell line T98G and U-25 IMG were obtained from the Molecular Toxicology Unit, Imperial College, London. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FCS, 15 mmol L⁻¹ L-glutamine, 100 units mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin in a humidified 5% CO₂ atmosphere at 37°C. For maintenance of the cells under serum-free conditions, the medium was replaced by the chemically defined MCDB 107 medium (Sigma).

DNA synthesis: Cells were seeded into 96–well microtiter plates (10⁶ cells/well) and cultured in DMEM, 10% FCS for

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12 h. Subsequently, cells were washed twice in serum-free MCDB 105 medium and grown for 24 h under serum-free conditions. The cells were finally incubated for another 48 h with various concentrations of tamoxifen (Sigma) (1 ng mL\(^{-1}\) to 10 µg mL\(^{-1}\)). Control cells were maintained in MCDB 105 without tamoxifen. [3H] thymidine (1.0 µCi/mL) was added for the last 12 h. Cells were washed with Phosphate-buffered Saline (PBS), detached by a 15 min incubation with 0.050/0.025% trypsin, 0.025% ethylene diaminetetra-acetic acid in PBS at 37°C and harvested onto filters. The filters were extensively washed and radioactivity was quantified in a liquid scintillation counter.

**Cell proliferation**: Cells were seeded into 96-well microtiter plates (5 x 10^3 cells/well) in serum supplemented medium. After overnight incubation, the medium were replaced with MCDB 105 and allowed to grow for 12 h. The medium were then changed into fresh MCDB 105 (as control) or into media containing tamoxifen at concentrations of 50 and 200 ng mL\(^{-1}\). Cells were detached with 0.25% trypsin in hanks balanced salt solution and counted in a hemocytometer at 48, 72, and 96 h. Cytostatic test was performed with 0.2% trypsin blue solution and the unstained viable cells were microscopically distinguished from the blue stained damaged cells.

**Tyrosine phosphorylation of neu receptor**: Cells were seeded in 10 cm tissue culture dishes and grown in DMEM containing 10% FCS. The cells were washed and incubated for 24 h with serum-free MCDB 105 medium. Various concentrations of tamoxifen (1 ng mL\(^{-1}\) to 10 µg mL\(^{-1}\)) were added and the dishes were incubated at 37°C for 10 min. The plates were washed with ice-cold PBS, including 100 µmol L\(^{-1}\) sodium orthovanadate. Cells were scraped into 0.5 mL of lysis buffer and lysates were centrifuged at 50,000 x g for 30 min at 4°C. Solubilized fractions of 1x 10^6 cells were incubated for 2 h with rabbit anti-neu antibody (a-neu, Oncogene Science, Inc). The immunoprecipitates were collected with Protein-A-Sepharose beads and washed with HNTG buffer (20 mmol L\(^{-1}\) 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid pH 7.5, 150 mmol L\(^{-1}\) NaCl, 10% glycerol, 0.1% Triton X-100) which was supplemented with 1 mmol L\(^{-1}\) sodium orthovanadate. The immunoprecipitated protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 7.5% gels and blotted with anti-phosphotyrosine antibody PY20 (1 µg mL\(^{-1}\) 2 h, Sigma). The tyrosine-phosphorylated proteins were detected using horseradish peroxidase (HRP) ABC kit (Sigma) and visualized with the ECL chemiluminescence method (Amersham Corp).

**Statistical analysis**: All experiments were performed three times. Statistical significance of thymidine uptake and cell growth was assessed by unpaired Student's t-test. All samples were done in triplicate.

**RESULTS**

**Effect on DNA synthesis**: [3H] thymidine uptake was reduced markedly in both U251-MG and T98G cell lines after exposure to 1 to 1000 ng mL\(^{-1}\) tamoxifen for 48 h (Fig. 1). The inhibition effect of tamoxifen displayed a dose-dependent pattern. At concentrations of tamoxifen greater than 200 ng mL\(^{-1}\) [3H] thymidine incorporation was less than 5% of control levels in two cell lines.

**Effect on cell proliferation**: After two cell lines were treated with 50 and 200 ng mL\(^{-1}\) tamoxifen, cell numbers were determined after 48, 72 and 96 h (Fig. 2). Cell growth was also inhibited by tamoxifen in a dose-dependent pattern. After 48 h, 50 ng mL\(^{-1}\) tamoxifen reduced cell numbers to less than 50% (42±3.0% of U251-MG and 39±2.5% of T98G) (p<0.001) and 200 ng mL\(^{-1}\) tamoxifen further reduced cell number to less than 25% (23±2.2% of U251-MG and 15±3.2% of T98G) (p<0.001) of the controls. At 200 ng mL\(^{-1}\) tamoxifen produced near complete inhibition of proliferation in each of the cell lines after 96 h. The effect achieved by this concentration was cytostatic rather than cytotoxic because more than 95% of cells excluded trypan blue.

**Effect on tyrosine phosphorylation of the neu receptor**: The interaction of tamoxifen with neu receptor in the cell lines was confirmed by assaying the receptor tyrosine phosphorylation after having treated it with 10, 50 and 200 ng mL\(^{-1}\) tamoxifen. Immunoprecipitation of neu receptor protein (185 kDa protein) from tamoxifen treated

**Fig. 1**: [3H] thymidine uptake in two human glioma cell lines (U251-MG, T98G) after exposure to tamoxifen (1 ng mL\(^{-1}\) to 10 µg mL\(^{-1}\)) for 12 h. Each point represents the average of triplicate determination.
inhibited and no phosphorylation was detected at 200 ng mL$^{-1}$ tamoxifen.

**DISCUSSION**

The PTK receptors are known to be involved in regulation of cell growth, cell differentiation, chemotaxis and actin reorganization\textsuperscript{5}. Phosphorylation of p185-neu on tyrosine could result from autophosphorylation or from phosphorylation by a distinct tyrosine kinase. Autophosphorylation of p185-neu could be activated by binding of a ligand to the external domain, or by clustering and subsequent intermolecular interactions of p185-neu monomers concentrated at high densities at the plasma membrane\textsuperscript{[13-17]}. In the present study the tyrosine phosphorylation of neu receptor as well as DNA synthesis and cell proliferation in the malignant glioma cell lines U25 l-MG and T98G was markedly inhibited by tamoxifen. The magnitude of this receptor phosphorylation correlated with the subsequent onset of DNA synthesis. These results suggest that tamoxifen interacts with neu receptor and inhibits tyrosine phosphorylation of neu receptor. It also shows that protein tyrosine phosphorylation of neu receptor is probably a critical factor in the proliferation of glioma. We infer the inhibition of neu receptor tyrosine phosphorylation resulted in blocking the signal transduction cascades from the receptor toward the DNA replication, proliferation and aggressive growth associated with malignancy\textsuperscript{[14]}. The anti-estrogen tamoxifen has long been used in the treatment of postmenopausal women with ER-positive breast carcinoma\textsuperscript{[6-7]} and is a potent inhibitor of the proliferation of ER-positive tumors in vitro. Studies showed that the effect of this agent and its metabolites in such tumors was largely mediated by competitive inhibition of estrogen binding to its receptor. Tamoxifen is also known to be a PKC inhibitor that effected cell proliferation by pathways independent of the ER\textsuperscript{[2,3,4,11-16]}.

The inhibition achieved by tamoxifen in these cell lines was demonstrated at concentrations that were achieved therapeutically in sera of patients with breast cancer (100 to 2000 ng mL$^{-1}$\textsuperscript{[12,17,18]}). Because tamoxifen can also penetrate the blood-brain barrier (BBB)\textsuperscript{[19]}, it has been successfully used in the treatment of cerebral metastases from breast cancer\textsuperscript{[19,20]} and to inhibit ER-mediated effects in various cell populations with the central nervous system that lie behind the BBB\textsuperscript{[20,21]} tamoxifen can be a potential agent in the treatment of malignant glioma. In conclusion, tamoxifen has proven to be a potent inhibitor of human glioma proliferation in vitro, possibly by inhibiting protein tyrosine (auto) phosphorylation in neu receptor. It has the potential to be
a useful agent in the treatment of glioma. Further research will carry on the mechanism of inhibition on the tyrosine phosphorylation in neu receptors.

REFERENCES


