Kobe J. Med. Sci. 47, 131 /140, June 2001

TAMOXIFEN INTERACTS WITH NEU/C-ERBB-2 RECEPTOR AND INHIBITS GROWTH OF HUMAN MALIGNANT GLIOMA CELL LINES

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KEY WORDS

tamoxifen; neu/c-erB-2; protein tyrosine phosphorylation; DNA; glioma

ABSTRACT

The 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. Pretreatment 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 correlatively. 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.

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). [4. 37)] 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.[19. 30)] Protein tyrosine kinase (PTK) receptors that have been implicated in a wide variety of physiological

Received for publication: January 29, 2001

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 tumor, PTK activity correlated with histology, lymph node metastasis, or prognosis.

Tamoxifen, (Z)-2[p-(1,2-diphenyl-1-butenyl) phenoxy]-N, N-dimethylethylamine citrate (1 : 1), a synthetic, nonsteroidal antiestrogenic compound, which has been used extensively in the treatment of estrogen receptor (ER)-positive breast cancer.[2, 16. 26)] Tamoxifen was reported to have inhibited the growth of some ER-negative cell lines of malignant glionla.[29. 32)] It has recently been reported that tamoxifen inhibits protein kinase C (PKC) by interfering with its activity of the catalytic subunit.[21 23. 32)] Tamoxifen can also penetrate the blood-brain barrier, [23)] which suggests a possible role for it in the treatment of malignant glionla.[29)]

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 Health Science Research Resources Bank. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FCS, 15mmol/L L-glutamine, 100 units/ml penicillin and 100μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37ºC. For maintenance of the cells under serum-free conditions, the medium was replaced by the chemically defined MCDB 105 medium (Sigma).

DNA synthesis

Cells were seeded into 96~well microtiter plates ($l0^4$ cells/well) and cultured in DMEM,10% FCS for 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) (1ng/ml to 10μg/ml). Control cells were maintained in MCDB 105 without tamoxifen. [^{3}H] thymidine (1.0μCi/ml) was added for the last 12 h. Cells were washed with phosphate-buffered saline (PBS), detached by a 15-minute

incubation with 0.050/0 trypsin, 0.02% ethylene diaminetetraacetic acid in PBS at 37ºC, 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 mediums were replaced with MCDB 105 and allowed to grow for 12 h. The mediums were then changed into fresh MCDB 105 (as control) or into mediums containing tamoxifen at concentrations of 50 and 200 ng/ml. Cells were detached with 0.25% trypsin in Hanks balanced salt solution and counted in a hemocytometer after 48, 72 and 96 h. Cytostatic test was performed with 0.2% trypan 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 (1ng/ml to 10 µg/ml) were added, and the dishes were incubated at 37ºC for 10 minutes. The plates were washed with ice-cold PBS, including 100µmol/L sodium orthovanadate. Cells were scraped into 0.5 ml of lysis buffer and lysates were centrifuged at 50,000 x g for 30 minutes at 4ºC. Solubilized fractions of 1x l06 cells were incubated for 2 h with rabbit anti-neu antibody (c-neu, Oncogene Science, Inc). The immunoprecipitates were collected with Protein A-Sepharose beads and washed with HNTG buffer (20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.5, 150 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100) which was supplemented with 1 mmol/L sodium orthovanadate. The immunoprecipitated protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 7.5% gels, and blotted with anti-phosphotyrosine antibody PY20 (1µg/ml 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

A11 experiments were performed three times. Statistical significance of thymidine uptake and cell growth was assessed by unpaired Student's t-test.

RESULTS

Effect on DNA synthesis

As shown in Fig. 1. [3H] thymidine uptake was reduced markedly in both U25 1-MG and T98G cell lines after exposure to 1 to 1000 ng/ml tamoxifen for 48h. The inhibition effect of tamoxifen displayed a dose-dependent pattern. At concentrations of tamoxifen greater than 200ng/ml [^{3}Hlthymidine incorporation was less than 5%. of control levels in two cell lines.

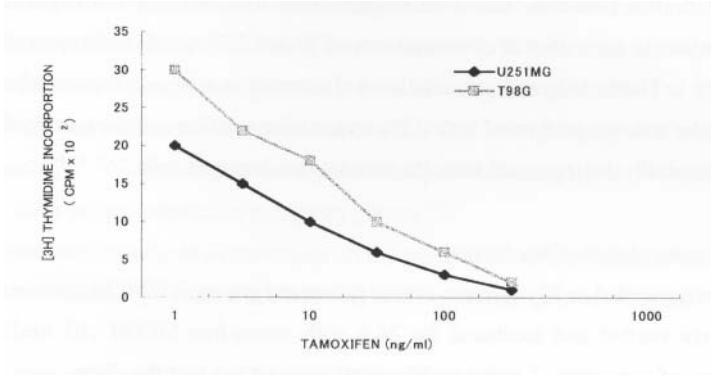

Fig.1.: Graph showing [^{3}H] thymidine uptake in two human glioma cell lines (U2S l-MG. T98G) after exposure to tamoxifen (1ng/ml to 10 µg/ml) for 12 h. Each point represents the average of triplicate determination.

Effect on cell proliferation

After two cell lines were treated with 50 and 200 ng/ml 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 48h, 50 ng/ml 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 tamoxifen further reduced cell number to less than 25% (23± 2% ofU251-MG and 15%± 3.2 of T98G) ($P<0.001$) of the controls. At 200 ng/ml 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.[32)]

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 ng/ml, 50 ng/ml and

200ng/ml tamoxifen. Immunoprecipitation of neu receptor protein (185-kDa protein) from tamoxifen treated cells was followed by immunoblotting with anti-phosphotyrosine antibody. Fig.3 shows that tyrosine phosphorylation of neu receptor protein in two cell lines was inhibited by various concentrations of tamoxifen in a dose-dependent pattern. The reduction of phosphorylated tyrosine protein, at 10, 50 and 200 ng/ml, was 3, 5 and 10-fold in U251MG cell, and it was 3,10 and 0-fold in T98G cell. In T98G cell line, the tyrosine phosphorylation was extremely inhibited and no phosphorylation was detected at 200 ng/ml tamoxifen.

DISSCUSSION

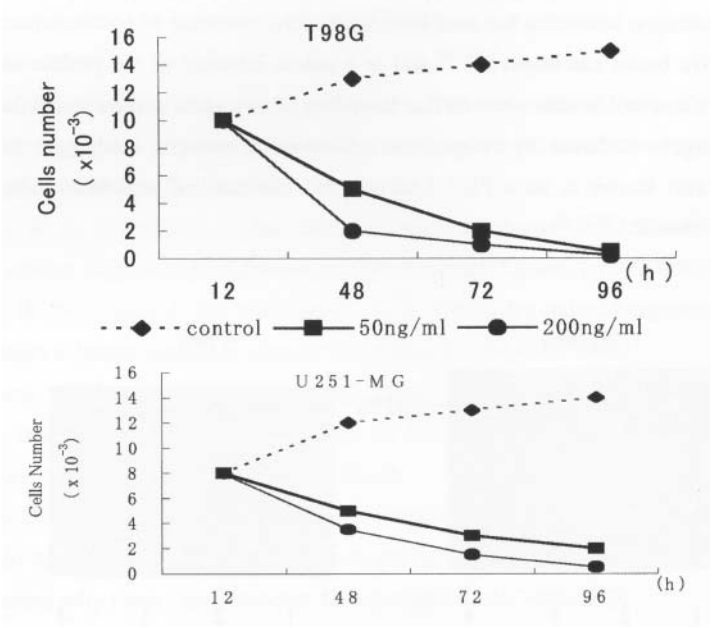

Fig.2.: Graphs showing cell growth curves in two glioma cell lines (U25 l-MG, T-98G) treated with tamoxifen in 50 ng/ml and 200 ng/ml for up to 96 hours.

The PTK receptors are known to be involved in regulation of cell growth cell differentiation, chemotaxis and actin reorganization.[20] 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 consequent intermolecular interactions of pl85-neu monomers concentrated at high densities at the plasma membrane.[8. 9, 34)]

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 transduetion cascades from the receptor toward the DNA replication Proliferation and aggressive growth associated with malignancy.[9).]

The anti-estrogen tamoxifen has long been used in the treatment of postmenopausal women with ER-positive breast carcinoma [2. 16, 26)] 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.[2. 23 25. 37)]

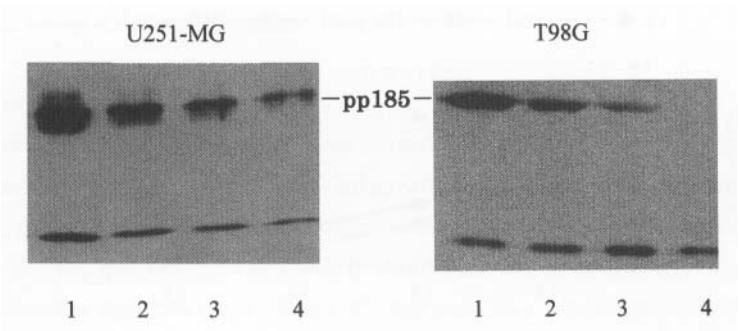

Fig. 3.: Tyrosine phosphorylation of the neu receptor. Cell lines U251-MG and T9gG were incubated without (lane 1) or with 10 ng/ml (lane 2), 50 ng/ml (lane 3), 200 ng/ml (lane 4) of tamoxifen for 10 min at 37°C. The neu receptor was immunoprecipitated with anti-neu receptor antibody and immunoblot analysis performed with anti-phosphotyrosine antibody. Positions of coelectrophoresed protein markers in kD are indicated.

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). [5. 17. 20)] Because tamoxifen can also penetrate the blood-brain barrier (BBB) [17)] it has been successfully used in the treatment of cerebral metastases from breast cancer [3.11)] and to inhibit ER-mediated effects in various cell populations with the central nervous system that lie behind the BBB, [7.11)] 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.

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