Anti-proliferative Effect of Toremifene and Tamoxifen on Estrogen Receptor-lacking Anaplastic Thyroid Carcinoma Cell Lines

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The antitumor effects of toremifene were studied *in vitro* using 10 anaplastic thyroid carcinoma cell lines and were compared with those of tamoxifen. The antitumor effect of toremifene was dose-dependent and the IC₅₀ values for these cell lines ranged 20.1–58.5 μ M. Although the cell lines expressed multidrug resistance gene mRNA, multidrug resistance-associated protein gene mRNA and insulin-like growth factor-1 (IGF-1) receptor mRNA to various degrees, nine of them lacked estrogen receptor expression as evaluated by immunocytochemistry. These data indicate that toremifene, as well as tamoxifen, is cytolytic at relatively high doses for multidrug-resistant anaplastic thyroid carcinoma cell lines.

Key words toremifene; anaplastic thyroid carcinoma; estrogen receptor; multidrug resistance; insulin-like growth factor-1 (IGF-1) receptor

Comparable clinical antitumor effects in breast cancers can be achieved with several hormonal treatments, including high-dose estrogen, high-dose progestin, anti-estrogen, oophorectomy, as well as surgical or chemical adrenalectomy.¹⁾ Tamoxifen, an antiestrogen antineoplastic drug, has been used extensively in the treatment of breast cancer, both in advanced cases and more recently as adjuvant treatment in early-stage disease.²⁾ Women treated with tamoxifen were found to have a lower incidence of secondary and contralateral breast cancer, so the possibility that tamoxifen could be an effective endocrine cancer preventive agent in women at increased risk of breast cancer has been proposed.^{3–5)}

Toremifene has also been developed as a breast cancer treatment.⁶⁾ It differs from tamoxifen only by the substitution of a chlorine atom for a hydrogen atom in an ethyl chain attached to one of the carbon atoms of the ethylene bond. Toremifene has antiestrogenic and estrogenic properties similar to those of tamoxifen.

Anaplastic thyroid carcinoma is one subtype of thyroid malignancy. The incidence of anaplastic thyroid carcinoma among thyroid neoplasms is very low, with less than 100 cases occurring annually in Japan.⁷⁾ The long-term survival rate of patients with this disease is still very low, although multimodal therapy, including intensive chemotherapy, has been used recently. Multidrug resistance mechanisms seem to be present in anaplastic thyroid carcinoma because the response to chemotherapy is very poor, regardless of whether there is rapid tumor progression.^{8,9)} We urgently need to find an antineoplastic drug that is effective against anaplastic thyroid carcinoma.

The present study was designed to examine the *in vitro* effects of toremifene and tamoxifen on anaplastic carcinoma of the thyroid, a hormone-producing organ.

MATERIALS AND METHODS

Cell Lines Ten anaplastic thyroid carcinoma cell lines (HTC/C3, KKS-2, KTA-2, MC3, TCO-1, TTA-1, TTA-3, 8305C, NTS-3 and TC78) and one breast cancer cell line,

MCF-7, were used in this study.¹⁰⁾

Growth-Inhibitory Effects (IC₅₀) of Toremifene and Tamoxifen on Cell Lines The IC₅₀ values were determined as described previously.¹⁰⁾ Briefly, cancer cells (3×10^5 /ml) were cultured at 37 °C for 5 h in 24-well culture plates containing 1.5 ml RPMI 1640 with 10% fetal calf serum. They were then treated with graded concentrations of toremifene (Nihon Kayaku Co., Tokyo, Japan) or tamoxifen (Nihon Kayaku Co., Tokyo, Japan) (0.1–100 μ M). The cells were cultured in the presence of the drug and were counted in a hemocytometer 3 d after drug treatment began. Three wells were used for each drug concentration. The IC₅₀ value was determined by plotting the logarithm of the drug concentration *versus* the growth rate (expressed as a percentage of the control) of the treated cells. The initial cell number was subtracted in the calculation.

Immunocytochemistry Immunocytochemistry using avidin–biotin complex-peroxidase (ABC-PO) and avidin–biotin complex-glucose oxidase (ABC-GO) was carried out as described elsewhere.^{11,12)} Anti-estrogen receptor monoclonal antibody Mab (Immunotech, Marseille, France), anti-progesterone receptor Mab (Immunotech), anti-insulin-like growth factor 1 (IGF-1) Mab (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.), and anti-IGF-1 receptor Mab (Calbiochem, Cambridge, Mass., U.S.A.) were used as the first antibodies. The optimal dilutions were 1:50, 1:50, 1:100 and 1:20, respectively. Immunoreactivity scoring was based on evaluation of cytospin preparations. Immunoreactivity was scored as negative when the cancer cells lacked immunostaining, and positive when they were immunostained. The immunoreactivity was reviewed by two expert immunopathologists, and similar results were obtained by each.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) RNA was extracted from the specimens using a standard method.¹³⁾ Five micrograms purified cellular RNA was converted to single-stranded cDNA using a random primer, and Moloney-MLV reverse transcriptase

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(Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) under reaction conditions described previously. The cDNAs homologous with the *mdr1*, *mrp*, IGF-1 receptor and β_2 -microglobulin gene-positive strand RNA were subjected to 30 amplification cycles, using an automated thermal cycler. The primers used in this study have been described previously except for the IGF-1 receptor gene primer sets. The cycle conditions included a 65°C annealing step for 5 min, a 72°C extension step for 5 min, and a 94°C denaturation step for 1 min. A 308-bp DNA fragment for the *mdr1*, a 399-bp DNA fragment for the *mrp*, a 261-bp DNA fragment for the β_2 -microglobulin gene and a 401-bp DNA fragment for the IGF-1 receptor gene were detected under these conditions. The IGF-1 receptor gene-specific primers used in this study were 5' ²⁹⁵⁶GTGTACGTTCTGATGAGT²⁹⁷⁵G 3' and 5' ³³³⁷TAAGTCTACCGGCCTCTCT³³⁵⁶A 3'. The intensity of mRNA expression was classified as either none observed (–) or strong (+), (the amplified band was less than half as intense as the band of half of the β_2 -microglobulin gene used as an internal control).¹⁴⁾

RESULTS

Antitumor Effects of Toremifene and Tamoxifen on Various Cell Lines The IC₅₀ values of toremifene against the anaplastic thyroid carcinoma cell lines are summarized in Table 1 in comparison with those of tamoxifen. The IC₅₀ was determined by constructing a dose-response curve of each drug as shown in Fig. 1. The drug concentration ranged from 20.1–58.5 μ M. Toremifene was effective against TTA-3, MC3, HTC/C3, KKS-2 and TCO-1 at relatively high doses. Toremifene was effective against KTA-2 and TTA-1 at relatively low doses compared with its IC₅₀ value for MCF-7 cells (10.1 μ M). Tamoxifen was also effective against these cell lines, but there were some differences in efficacy of toremifene and tamoxifen for TC78, NTS-3, KKS-2 and TCO-1 (IC₅₀ in μ M: 41.8 vs. 16.7, 30.1 vs. 16.3, 53.5 vs. 18.6, and 55.2 vs. 22.2), respectively. The IC₅₀ of tamoxifen ranged from 16.3–40.8 μ M.

Immunocytochemistry Estrogen receptors were expressed only by KKS-2 cells (Table 2). The other nine

anaplastic thyroid carcinoma cell lines were negative for estrogen receptors (Table 2). Five cell lines (TTA-1, TC78, KTA-2, KKS-2 and 8305C) were positive for anti-progesterone receptor Mab staining. Four cell lines (TTA-3, KTA-2, KKS-2 and 8305C) were positive for anti-IGF-1 Mab staining and nine cell lines, all except MC3, expressed IGF-1 receptors intensely (Fig. 2 and Table 2).

RT-PCR *Mdr1* mRNA was expressed at significant

Fig. 1. Representative Dose-Response Curves of Tamoxifen (A) and Toremifene (B) against MC3

X-axis shows drug concentration (μ g/ml) and Y-axis shows cell survival ratio (%).

Table 1. Fifty Percent Growth-inhibitory Concentration (IC₅₀) of Toremifene and Tamoxifen against Various Cell Lines

Cell lines	IC ₅₀ (μ M)	
	Toremifene	Tamoxifen
TTA-1	25.9	22.2
TTA-3	55.2	40.8
TC78	41.8	16.7
NTS-3	30.1	16.3
KTA-2	20.1	19.5
MC3	41.9	16.7
HTC/C3	55.2	30.2
KKS-2	53.5	18.6
8305C	48.5	28.4
TCO-1	55.2	22.2
MCF-7	10.1	6.3

Table 2. Expression of Estrogen Receptors (ER), Progesterone Receptors (PR), IGF-1, and IGF-1 Receptors in Various Cell Lines

Cell line	Immunocytochemistry			
	ER	PR	IGF-1	IGF-1 R
TTA-1	–	+	–	+
TTA-3	–	–	+	+
TC78	–	+	–	+
NTS-3	–	–	–	+
KTA-2	–	+	+	+
MC3	–	–	ND	ND
HTC/C3	–	–	–	+
KKS-2	+	+	+	+
8305C	–	+	+	+
TCO-1	–	–	–	+
MCF-7	+	+	+	+

ND: not determined.

Fig. 2. Immunostaining of Anaplastic Thyroid Carcinoma Cells with Anti-estrogen Receptor Mab (A, TC78), Anti-IGF-1 Mab (B, KKS-2) and Anti-IGF-1 Receptor Mab (C, TC78)

× 100. ABC-PO method. Counterstaining with hematoxylin. TC78 cells do not express estrogen receptors, but they express IGF-1 receptors significantly in their cytoplasm (A and C). KKS-2 cells express IGF-1 in their cytoplasm (B).

levels in TTA-1, TTA-3, TC78, HTC/C3, KKS-2, 8305C and TCO-1 (Table 3). All the cell lines expressed significant levels of mrp mRNA. These results were in agreement with previous reports. Nine out of ten anaplastic thyroid carcinoma cell lines expressed significant levels of IGF-1 receptor mRNA, as shown in Fig. 3. MCF-7 cells used as a control did not express mdrl and mrp mRNA.

DISCUSSION

We found that toremifene, as well as tamoxifen, showed antitumor effects on anaplastic thyroid carcinoma cell lines *in vitro*. There are three points about this that warrant discussion.

Firstly, toremifene and tamoxifen were effective against anaplastic carcinoma cell lines of the thyroid which did not express significant levels of estrogen receptors. Only KKS-2 cells expressed estrogen receptors as evaluated by immunocytochemistry and RT-PCR (data not shown). The basic pharmacological mechanism of action of toremifene resembles that of tamoxifen.⁶⁾ Both compounds bind to estrogen receptors and are antiestrogenic in that they inhibit estradiol-induced uterotrophic effects in mice, rats, and humans.⁶⁾ At high dose levels *in vivo* toremifene seems to exert an antitumor effect which cannot be explained by antiestrogenic characteristics as our data have shown. Toremifene and tamoxifen were not cytotoxic to the anaplastic thyroid carcinoma cell lines at concentra-

Table 3. Mdr1, mrp and IGF-1 R mRNA Expression by RT-PCR

Cell line	RT-PCR		
	mdr1	mrp	IGF-1 R
TTA-1	+	+	+
TTA-3	+	+	+
TC78	+	+	+
NTS-3	—	+	+
KTA-2	—	+	+
MC3	—	+	—
HTC/C3	+	+	+
KKS-2	+	+	+
8305C	+	+	+
TCO-1	+	+	+
MCF-7	—	—	+

Refer to Materials and Methods for judgment criteria.

Fig. 3. RT-PCR Products Separated in 2% Agarose Gel

TTA-3, KKS-2, NTS-3, KTA-2, HTC/C3 and 8305C express IGF-1 receptor mRNA strongly and TTA-1, TCO-1 and TC78 express IGF-1 receptor mRNA weakly. MC3 lacks IGF-1 receptor mRNA.

tions expressed as IC_{50} . They were toxic to the cell lines at much higher doses. In fact, tamoxifen inhibits the growth of estrogen receptor-negative A549 lung cancer cells.¹⁵⁾ Sutherland *et al.* have suggested that binding to specific cytosolic antiestrogen binding sites is essential for this cytotoxic effect.¹⁶⁾ Although there are no experimental data indicating that toremifene binds to antiestrogenic binding sites, this hypothesis is in agreement with our results, which show an antitumor effect of toremifene *in vitro* against human anaplastic thyroid carcinoma cell lines, which did not express estrogen receptors at protein and mRNA levels. Thus, toremifene seems to have antitumor or cytolytic effects, which appear at high doses and which may not be dependent on estrogen receptors.¹⁵⁾

Secondly, the anaplastic thyroid carcinoma cell lines expressed MDR1 and/or MRP to various degrees and this multidrug resistance may be explained in part by the high doses of toremifene and tamoxifen used. Tamoxifen reverses multidrug resistance,¹⁷⁾ while tamoxifen selectively inhibits chloride channel activity.¹⁸⁾ Thus, tamoxifen circumvents multidrug resistance in human gastrointestinal tumors, leukemia and other conditions.^{19,20)} It has been reported that our cell lines are resistant to doxorubicin, and that acquired resistance to doxorubicin in P388 murine leukemia cells is reversed by tamoxifen. So toremifene may have a similar effect.^{10,21)} It is of great interest that exposure to toremifene alone, or to tamoxifen, has an antitumor effect on multidrug resistant tumor cell lines.

Lastly, our cell lines expressed significant levels of IGF-I and IGF-I receptors. Although we did not detect a significant decrease in IGF-I levels in the presence of tamoxifen (data not shown), *in vitro* inhibition of IGF-I gene expression was induced by toremifene, as has been previously reported for tamoxifen.²²⁾ Consequently, tamoxifen and toremifene may have antineoplastic activity because IGF-I is a potent mitogen for tumor cells.²³⁾ Alternatively, toremifene may exert its antitumor effect through IGF-I receptors because, although these cell lines did not express estrogen receptors, they did express significant levels of IGF-I receptors.

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