Antagonist effect of insulin-like growth factor I on protein kinase inhibitor–mediated apoptosis in human glioblastoma cells in association with bcl-2 and bcl-xL

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Object. Tamoxifen (TAM) has been found to be effective in inhibiting proliferation of glioblastoma cells in vitro, but clinical studies have been disappointing. The purpose of this study was to determine whether insulin-like growth factor I (IGF-I), a potential autocrine/paracrine mitogen produced by glioblastomas, interferes with the antimitogenic actions of TAM.

Methods. Human glioblastoma cells were treated with or without TAM and/or IGF-I in vitro and evaluated for: viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide cleavage assay; apoptosis by histochemical analysis of nuclear morphology and 3'-OH DNA fragments; and expression of the IGF-I receptor, and the bcl-2, bcl-xL, and bax proteins by immunoblot analysis. In addition, p53 status was determined by DNA sequencing and by transient transfection with luciferase reporter plasmids containing wild-type or mutant p53. Results indicated that after 72 hours of exposure to 2 mg/ml TAM in vitro, 56.3% of WITG3 and 43.8% of U87-MG glioblastoma cells contained apoptotic nuclei (p < 0.01 compared with untreated cells). Apoptosis was independent of the presence of p53 because the WITG3 cells, in contrast to the U87-MG cells, expressed a mutant, nonfunctional p53. The WITG3 cells expressed IGF-I receptor proteins and demonstrated IGF-I binding. Exogenous IGF-I stimulated WITG3 cell proliferation and significantly (p < 0.05) antagonized the cytotoxic effects of TAM in a dose-dependent fashion; IGF-I, but not TAM, enhanced expression of bcl-2 and bcl-xL proteins; however, bax protein expression was unchanged by either treatment.

Conclusions. Because many gliomas secrete large amounts of IGF-I in autocrine/paracrine growth pathways, these data may, in part, explain the failure of TAM to achieve clinical results as dramatic as those in vitro.

Key Words • tamoxifen • glioblastoma multiforme • apoptosis • insulin-like growth factor I

Despite advances in the understanding of the molecular biology of glial neoplasms, the prognosis for patients with glioblastoma multiforme remains dismal. Over the last several years, researchers in several laboratories have reported that modulation of the protein kinase C (PKC) pathways inhibited growth of malignant glioma cell lines3–5,23 and even mediated programmed cell death.4 Tamoxifen (TAM) is a nonsteroidal estrogen antagonist that also has been demonstrated to inhibit PKC3,5,22,23.

We have focused on the role of TAM in the inhibition of invasion and proliferation of glioblastoma cells by using the human glioblastoma cell line, WITG3.12 Preliminary studies suggested that TAM could induce apoptosis in glioma cell lines independent of estrogen receptor status.12 However, these promising in vitro findings seemed inconsistent with clinical reports that indicate that administration of TAM in doses comparable to those used in the laboratory had only limited clinical effectiveness.6

Insulin-like growth factor I (IGF-I) is a mitogenic polypeptide that is important as both an endocrine and paracrine/autocrine growth factor.16 Binding of IGF-I to its receptor activates its tyrosine phosphorylation capacity. The activated receptor is then capable of phosphorylating other proteins and mediating signaling through mitogen-activated protein kinase (MAPK) signal transduction pathways. It is believed that signaling through MAPK pathways initiates the transcription of new genes involved in growth stimulation and progression through the cell cycle.21

Tamoxifen has been shown to inhibit gene expression of IGF-I in vivo11 and to reduce serum levels of IGF-I in breast cancer.15 Insulin-like growth factor I has been observed to inhibit apoptosis of erythroid colonies,18 breast cancer cells,7 and cerebellar granule neurons.9 Insulin-like growth factor I has also been noted to be an important mitogen in modulating growth of glioblastomas multiforme and to be highly expressed in many malignant glial
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neoplasms. Based on such evidence, we hypothesized that IGF-I might in some way interfere with the actions of TAM and thus explain both the in vitro efficacy of TAM as well as its disappointing performance in vivo.

Materials and Methods

Cell Culture

The WITG3 is a glioblastoma cell line derived from a human surgical specimen and has been characterized in detail previously. Cultured cells were grown in RPMI-1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS), 1-glutamine, and penicillin-streptomycin at 37°C in a humidified atmosphere (95% air/5% CO2), unless otherwise noted. The U87-MG cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1-glutamine, and penicillin-streptomycin. Evaluation of IGF-I levels in serum-free conditioned medium by immunoradiometric assay was performed by the laboratory of Dr. Manjula Gupta, Department of Clinical Pathology, The Cleveland Clinic Foundation.

Reverse Transcriptase–Polymerase Chain Reaction Amplification and DNA Sequencing

Reverse transcription was performed in 1 to 2 μg of total RNA, followed by polymerase chain reaction (PCR) amplification and sequencing. The p53 was amplified as two fragments by using primers P53S1-SQ3 and SQ1-P53AS. This results in two overlapping PCR fragments of 696 bp and 941 bp, respectively, that encompass the entire p53 coding region. The PCR products were purified using purification columns. Following PCR cycle sequencing, sequences were resolved on an automated DNA sequencer. Samples were sequenced using primers S1 (for the P53S1:SQ3 fragments) or SQ1, SQ9, SQ17, and SQ19 (for the SQ1:P53AS fragments). Mutations were confirmed using reverse-strand sequence primers previously described. Sequences were analyzed and compared with the wild-type p53 sequence of U87-MG.

Evaluation of p53 Activity by Luciferase Reporter Plasmids

The luciferase reporter plasmids PG13 and MG15 are constructs modeled after the PG13 (wild-type p53 binding) and MG15 (p53 binding-site mutation) chloramphenicol acetyltransferase reporter plasmids. Briefly, these constructs contain multiple copies of the wild type (PG13) or mutant (MG15) p53 binding sequences located immediately upstream of the early gene promoter of the Polyoma virus. Downstream of this promoter is the luciferase reporter gene. The WITG3 and U87-MG cells were plated at a density of 500,000 cells/ml in Petri dishes. The cells were incubated overnight; the cells were washed and the media were replaced with OPTI-MEM.

Hoechst Staining

After culturing cells overnight to allow for cell adherence, the cells were washed twice with PBS and incubated for 24 to 72 hours in 1% FBS-supplemented medium with or without IGF-I or TAM. Floating cells were collected and adherent cells were trypsinized and pelleted. The entire cell suspension (floating plus adherent cells) was fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 minutes. The cell suspension (200 μl) was dried on a microscope slide and stained with Hoechst 33258 for 30 minutes. Slides were washed three times in PBS and examined by ultraviolet microscopy. One hundred nuclei were counted for each data point in five replicates per point. The cells were evaluated for signs of nuclear fragmentation and condensation, which are characteristic of apoptosis.

Additional TAM-treated cell suspensions were fixed in 4% buffered formalin and treated with terminal deoxynucleotidyl transferase plus digoxigenin-nucleotide for detection of 3'OH DNA fragments by using the Apotag histochemical method. Hydrogen peroxide dianisobenzidine substrate solution was applied to allow color development, and the slides were washed and counterstained in 0.5% methyl green.

Protein Isolation and Immunoblot Analysis

The cells were disrupted in cold lysis buffer (10 mM tetra sodium pyrophosphate, 20 mM HEPES, 1 Triton X-100, 100 mM NaCl, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride). The crude lysates were cleared by centrifugation and protein concentrations were determined by using the Bradford protein assay. Equal amounts of protein were placed in 2× sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 0.2 mg/ml bromophenol blue dye, 2% sodium dodecyl sulfate [SDS], 10% β-mercapto-ethanol) and electrophoresed on a 10% SDS–polyacrylamide electrophoretic gel. The proteins were then transferred to a nitrocellulose membrane by using an electrobonding technique. Membranes were blocked for 1 hour at room temperature in Tris buffer saline with Tween (TBST) and 5% nonfat milk. Primary antibodies to bcl-2, bcl-xL, and bax were incubated for 1 hour at room temperature in TBST and 1% nonfat milk. The blots were then washed and incubated with a peroxidase-conjugated secondary antibody for 1 hour in TBST. The chemiluminescent substrate for the secondary antibody was developed with a detection system. Blots were exposed for 3 to 5 minutes to film and developed.

Statistical Analysis

Results were analyzed for statistical significance by means of the Student t-test for paired data and the Tukey–Kramer test for multiple comparisons by using commercially available software.
FIG. 1. Bar graphs. Upper: Viability of WITG3 and U87-MG cells treated with TAM. The relative percentage of viability was determined by the MTT assay. Results in cells treated with all concentrations of TAM were significantly (p < 0.001) different from results in untreated control cells. Lower: Effect of TAM or irradiation on the activation of p53 in U87-MG and WITG3 glioblastoma cells as determined by the luciferase assay. Cells were transiently transfected with either plasmid PG13 (wild-type p53 binding sequence) or plasmid MG15 (mutant p53 binding sequence). Activation is placed on the graph as the log-fold increase in luciferase activity relative to controls in which 1.0 equals either cell line transfected with PG13 plasmid and cultured without 5 Gy irradiation or 2 μg/ml TAM treatment.

Sources of Supplies and Equipment

GIBCO (Grand Island, NY) produced the FBS, 1-glutamine, penicillin-streptomycin, lipofectamine, OPTI-MEM, and Hank’s balanced salt solution. GIBCO BRL (Gaithersburg, MD) produced the IGF-I. The [125I]-IGF-I and the MTT cleavage assay kit were purchased from Boehringer Mannheim (Indianapolis, IN). The TAM and Hoechst 33258 were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide diaminobenzidine substrate solution was obtained from Dako Corp. (Carpinteria, CA).

The U87-MG cells were obtained from Riken Cell Bank (Wako, Japan). The QiAquick PCR purification columns were purchased from Qiagen (Carpenteria, CA) and used to purify the PCR products; sequences were resolved on an ABI 373A automated DNA sequencer purchased from Perkin Elmer (Norwalk, CT) and analyzed by using the Sequence Navigator program available from Applied Biosystems, Inc. (Norwalk, CT).

Examination of cells for protein content and luciferase activity was accomplished by using luciferase assay reagents obtained from Promega (Madison, WI). A microtiter plate luminometer obtained from Dynatech Laboratories, Inc. (Chantilly, VA) was used to measure transcription of the luciferase gene.

Bio-Rad Laboratories (Richmond, CA) provided the gamma counter and reagents used in the Bradford protein assay. The ELISA plate reader was obtained from Molecular Devices (Menlo Park, CA). Primary antibodies to bcl-2, bcl-xl, and bax were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL detection system used to develop the chemiluminescent substrate for the secondary antibody was purchased from Amersham (Arlington Heights, IL).

Film used in the immunoblot analysis was obtained from Eastman Kodak Co. (Rochester, NY). GraphPad InStat software available from GraphPad (San Diego, CA) was used to perform the statistical analysis.

Results

Tamoxifen-Induced Apoptosis Independent of p53 Status

The MTT assays indicated that TAM (0.5–4 μg/ml) significantly (p < 0.001) reduced viability in WITG3 and U87-MG cells in a dose-dependent manner (Fig. 1 upper). By using Hoechst staining, apoptotic nuclear morphology was evident at 72 hours in 56.3% of TAM-treated (2 μg/ml) WITG3 cells compared with 1.5% of untreated control cells (p < 0.01). The presence of apoptosis-associated DNA damage was confirmed by detection of 3′-OH DNA labeling; this was evident as peroxidase-labeled nuclei in 49.1% of TAM-treated cells compared with 1% of control (p < 0.01). The DNA laddering was not consistently found (data not shown), but these results may be in line with those of Oberhammer, et al., who reported that not all types of apoptosis produced internucleosomal fragments yielding DNA laddering. Primary necrotic death was not observed after TAM treatment, as noted previously.

In U87-MG cells, which have been shown to carry wild-type p53, apoptotic nuclei were evident by Hoechst staining in 43.8% of TAM-treated (0.5 mg/ml TAM) cells compared to 2% of untreated controls (p < 0.01). Sequence analysis of WITG3 p53 DNA revealed an adenine-to-guanine mutation in codon 242 resulting in a methionine-to-valine mutation in the protein. Sequencing with a dideoxy chain termination methodology confirmed that no wild-type p53 was present in the WITG3 cells. The mutation occurred in one of the functionally conserved domains of p53 adjacent to a zinc finger-binding motif. The location and frequency of mutations in this region in tumors suggests that changes in the ability to bind DNA with the zinc finger causes a loss of p53 function.

To examine the function of WITG3 p53, both U87-MG (used as a wild-type p53 control) and WITG3 cells were transfected with either the MG15 plasmid (mutant p53 binding sequence) or the PG13 plasmid (containing a normal p53 binding construct). Because the DNA damage induced by irradiation is recognized as a p53 activation event, the cells were irradiated with a single 5-Gy dose or exposed to TAM. Irradiated U87-MG/PG13 cells had a relative increase of 41.7 over untreated, 24-hour-cultured U87-MG/PG13 cells or irradiated U87-MG/MG15 cells. In contrast, the luciferase activity of WITG3/MG13 cells did not change relative to the untreated control or WITG3/MG15 cells (Fig. 1 lower). Treatment of either WITG3 or U87-MG cells with TAM (2 μg/ml and 0.5 μg/ml, respectively) showed no activation of p53 (Fig. 1 lower). These doses were based on MTT viability data that indicated that higher doses might kill the majority of the cells during the period of evaluation for the luciferase assay.
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These data showed that WITG3 cells, in contrast to U87-MG cells, could not bind the normal p53 consensus binding sequence in the PG13 plasmid and activate the luciferase reporter gene in response to radiation, confirming the presence of a nonfunctional p53. Tamoxifen did not induce activation of the luciferase reporter in either U87-MG or WITG3 cells, suggesting that its action was independent of the presence of p53.

Evidence for Functional IGF-I Receptors

Examination of WITG3 cells for evidence of IGF-I receptors was performed by means of an immunoblotting and radioreceptor assay. Immunoblots indicated the presence of both 98-kD and 130-kD IGF-I receptor subunits (Fig. 2). The receptors were more highly expressed in cells cultured either in 10% FBS (which represents a mixture of growth factors and growth-promoting substances) or in 10 nM IGF-I, suggesting that receptor levels were regulated by the presence of substrate (Fig. 2). The \(^{125}\)I-IGF-I binding assays illustrated competitive binding of radiolabeled substrate, which was compatible with the presence of a functional IGF-I binding domain (data not shown). The numbers of binding sites per cell averaged $4.5 \times 10^4$ and are comparable to numbers of IGF-I binding sites reported in other types of human tumor cells. Basal IGF-I production by WITG3 cells was suggested by immunoradiometric results indicating $193 \pm 49$ pg/ml IGF-I in 24-hour conditioned medium from WITG3 cells compared with less than 27 pg/ml in medium alone.

Fig. 2. Results of immunoblot analysis of IGF-I receptors (IGF-IR) in WITG3 whole-cell lysates after culture in serum-free (SF) conditions for 6 and 12 hours with or without 10 nM IGF-I (I), 2 \(\mu\)g/ml TAM (T), or a combination of both (I+T). Control cells were cultured in 10% FBS. Both 130-kD and 98-kD subunits of the IGF-I receptor are visible.

Fig. 3. Bar graph displaying viability of WITG3 cells treated with TAM with or without IGF-I as measured by the MTT assay.

Fig. 4. Photomicrographs showing WITG3 cells grown in serum-free medium with 2 \(\mu\)g/ml TAM with or without 10 nM IGF-I for 72 hours. A: Control cells in medium. B: Cells in medium with TAM alone. C: Cells in medium with both TAM and IGF-I. Arrows indicate apoptotic cells. Hoecht 33258 stain, original magnification $\times 400$. 

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In WITG3 cells treated with TAM, a dose-dependent increase in viability was noted in the presence of exogenous IGF-I (Fig. 3). In cells treated for 72 hours with 2 µg/ml TAM, the addition of 5 nM IGF-I increased cell viability from 47 to 70% (p < 0.01). Supplementation with 10 nM IGF-I further increased viability to 79% (p < 0.001). Additional experiments (data not shown) also confirmed that IGF-I alone (at 10–14 nM) significantly (p < 0.01) augmented survival of WITG3 cells in serum-free medium by 32 ± 9% (three experiments), suggesting that IGF-I enhanced cell proliferation. Hoechst 33258 staining confirmed the presence of fewer apoptotic cells after a 72-hour treatment with TAM and IGF-I (32.4%, p < 0.05) compared with treatment with TAM alone (78.4%) (Fig. 4).

Augmentation of Expression of bcl-2 and bcl-xL Proteins by IGF-I

Lysates of WITG3 cells grown in 10% FBS showed a high basal expression of bcl-xL and a low level of bcl-2 protein (Fig. 5). Serum deprivation caused a drop in the expression of these proteins within 6 hours; however, levels were restored by 12 hours. In contrast, cells treated with 10 nM IGF-I maintained an expression of bcl-xL equal to that of serum-containing control at 6 and 12 hours. The IGF-I also increased bcl-2 expression over that of 6-hour serum-free controls. In serum-free medium containing TAM (2 µg/ml), expression of both bcl-2 and bcl-xL remained at low levels and the proteins were virtually undetectable by 12 hours. After 12 hours of exposure to IGF-I plus TAM, however, high levels of both proteins were maintained, resembling those of cells treated with IGF-I alone (Fig. 5).

Expression of bax Unchanged by TAM-Induced Apoptosis

The closely related proteins, bcl-2 and bax are capable of forming both homo- and heterodimers. It is thought that the relative ratio of bcl-2 to bax may predict whether cells are likely to undergo apoptosis or survive.27 In the WITG3 cell line, there was a high basal expression of bax protein; this did not change in response to serum deprivation or to treatment with TAM or IGF-I (Fig. 5).

Discussion

Previous studies have shown that PKC inhibitors indicate. The schism between the results of experiments in which cultured cells are used and the clinical efficacy of TAM led us to consider alternative hypotheses for the failure of TAM in vivo. Previously IGF-I was reported to be an important autocrine/paracrine growth factor commonly expressed in malignant glioma.24 Moreover, IGF-I has been found to inhibit apoptosis in diverse cell types.7,9,20,25 We have shown that the glioma cell line WITG3 has intact IGF-I receptors and that IGF-I is mitogenic for these cells. The addition of exogenous IGF-I inhibits apoptosis in association with an increase in the cell survival proteins bcl-2 and bcl-xL. Such results are compatible with observations by Parrizas and LeRoith,20 who noted that IGF-I augmented bcl-xL expression while inhibiting apoptosis in serum-deprived PC-12 cells. Not surprisingly, the p53-dependent, bcl-2 family member, bax,19 was not regulated in the WITG3 cell line, which contains mutant p53.

These results suggest that gliomas may escape cell death in vitro by an IGF-I–mediated increase in bcl-2 and bcl-xL. We propose that gliomas in vivo might escape cell death induced by TAM if they overexpress large amounts of IGF-I. Therefore, the discouraging clinical results reported thus far with TAM may be mediated by mechanisms similar to those demonstrated in this investigation. Because IGF-I signaling occurs via MAPK pathways and IGF-I supplementation leads to increased expression of the survival proteins bcl-2 and bcl-xL, this suggests that the blockade of MAPK pathways may increase the efficacy of TAM. It thus appears that additional research into the molecular biological characteristics and intracellular signaling of gliomas may improve our knowledge and lead to a more rational design of therapy for these neoplasms.

References

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