ABSTRACT

Cholangiocarcinoma represents a challenging primary malignancy of the liver with no effective medical therapy and a poor prognosis. We have investigated the role of tamoxifen and estrogen receptors (ERs) in the regulation of growth of human cholangiocarcinoma. Two human cholangiocarcinoma cell lines, OZ and SK-ChA-1, were grown in the presence of graded concentrations of tamoxifen; the effects on cell growth were determined by cell counting or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium proliferation assay. The presence of ER protein was tested by indirect immunofluorescence and immunoprecipitation. Reverse transcription-PCR was performed to confirm the presence of ER messenger RNA (mRNA) in both cell lines. Northern blot analysis confirmed the presence of full-length 6.5-kb ER mRNA. No ER detection mutants were detected. Tamoxifen inhibited the growth of human cholangiocarcinoma in vitro and in vivo. ER protein and mRNA were detected in both cell lines. The mechanism(s) of tamoxifen-mediated growth inhibition is unclear but may occur via ER protein or additional pathways. The ability of tamoxifen to inhibit tumor growth may offer an alternative adjunctive treatment for cholangiocarcinoma.

INTRODUCTION

Cholangiocarcinoma is a malignant tumor of the biliary tree that originates from the bile duct epithelial cell or cholangiocyte. Cholangiocarcinoma is an increasingly frequent diagnosis worldwide; in the United States, approximately 3000 new cases are reported each year. There is currently no effective medical, chemotherapeutic, or radiation therapy available; thus, the disease has a poor prognosis (1–5).

The etiology of cholangiocarcinoma is unknown; however, several well-described associations are reported as increased risks for the development of cholangiocarcinoma. These include chronic liver fluke infestation, exposure to various chemicals, and certain congenital diseases of the biliary tree (1, 4, 6–8). The association of primary sclerosing cholangitis (PSC) with cholangiocarcinoma is also now well recognized (1, 4, 9). The common underlying theme for these associations is that chronic inflammation of the biliary tree leads to an increased risk of cholangiocarcinoma development.

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2 To whom requests for reprints should be addressed, at University of Alabama at Birmingham, 405 Krakke Building, 1922 Seventh Avenue, South, Birmingham, AL 35294-0005.

3 The abbreviations used are: PSC, primary sclerosing cholangitis; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; TGF, transforming growth factor.

Tamoxifen, an anti-estrogen, has clear efficacy in the treatment of women with ER-positive breast cancer (10, 11). In addition, subgroups of ER-negative breast cancer patients also respond to tamoxifen therapy (11, 12). Recent studies have also shown that tamoxifen has efficacy in the treatment of central nervous system tumors such as malignant gliomas and craniopharyngiomas (13–15). On the basis of the poor survival record and the lack of effective therapy for cholangiocarcinoma and our desire to find an alternative form of therapy for biliary tract malignancy, we investigated the role of tamoxifen and its potential efficacy in slowing the growth of human cholangiocarcinoma cell lines. The purpose of this investigation was to address whether tamoxifen inhibits the in vitro growth of human cholangiocarcinoma and to initiate studies on the mechanism(s) of tumor growth inhibition.

MATERIALS AND METHODS

Cell Lines. Two separately derived human cholangiocarcinoma tumor cell lines, designated OZ and SK-ChA-1, were provided by Dr. N. F. LaRusso (Mayo Clinic, Rochester, MN) and Dr. A. Knuth (Ludwig Institute for Cancer Research, London, United Kingdom). The original characterization of these cell lines has been described (16, 17). MCF-7 (human breast cancer cell line), HEP-G2 (a human hepatoma cell line), PANC-1 (a human pancreatic cancer cell line), and COS cells (negative control) were purchased from the American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI 1640 (Cellgro; Fisher Scientific, Atlanta, GA) supplemented with 2% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). For the colorimetric proliferation assay, cells were plated at various times.

Cell Proline Assay. Cell proliferation was assessed by direct cell counting or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (CellTiter 96AQueous assay; Promega Corp., Madison, WI). For the colorimetric proliferation assay, cells were plated in quadruplicate into 96-well tissue culture plates (Nunc; Fischer Scientific) at 5–10,000 cells/well in a final volume of 200 μL. Cells were allowed to adhere overnight, medium containing various inhibitors was added, and cell counts were performed at various times.

Estradiol Stimulation of Cell Growth. Cell lines were maintained in estrogen-depleted medium (phenol red-free RPMI 1640 and charcoal-treated FCS) for 7 days before seeding at 5 × 10^5 cells/well (24-well plates; Nunc) in estrogen-depleted medium. Cells were stimulated with 10^-9, 10^-10, and 10^-11 M estradiol for 7 days (performed in triplicate). Controls included a carrier control alone without estradiol (methanol) and FCS that had not been treated...
with charcoal. After 1 week, cells were collected by trypsinization and counted on a Coulter counter. Results are expressed as the mean cell number with unstimulated control cells grown in estrogen-depleted medium normalized to 100%.

**RNA Extraction.** Total RNA was extracted from cell lines by the single-step method (19). Briefly, cultured cells were homogenized in a denaturing solution containing 4 m guanidinium thiocyanate followed by mixing with 0.1X volume 2 M sodium acetate, pH 4.0. An equal volume of phenol was added, vortexed, and followed with 0.2X volume of chloroform/isoamyl alcohol. The resulting mixture was centrifuged, and the upper aqueous phase was precipitated with isopropanol and washed with 70% ethanol. RNA concentration was determined spectrophotometrically.

**RT-PCR.** Total RNA (1 μg) was reverse-transcribed in the presence of 1 mM dTTPs (Promega), 20 μg oligo(dT) primers, 10 mm DTT, and 5 units reverse transcriptase/ml (Life Technologies, Inc., Grand Island, NY) for 10 min at 65°C, 60 min at 42°C, and 5 min at 90°C. Three pairs of primers were selected that spanned either the DNA-binding region (exons 2 and 3, primers 1 and 2) or part of the ligand-binding region (exon 5, primers 3 and 4; and exon 6, primers 5 and 6) of the ER (18). ER cDNA was amplified using Taq polymerase (Promega) for 40 cycles; each cycle included one cycle at 44°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplified DNA fragments were then analyzed by a 2% agarose gel.

**Northern Blot Analysis.** Northern blot analysis was performed essentially as described (19). Briefly, total RNA (1.0 mg) was electrophoresed through a formaldehyde-agarose gel, blotted onto a nylon membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked. Hybridization was performed in a Techne Hybridiser HB-10 for 60 min at 68°C using a [32P]dATP-labeled ER probe in 15 ml of Quik Hyb solution (Stratagene). Plasmid ER cDNA was isolated with a Qiagen kit (Chatsworth, CA) and digested with EcoRI (Promega); the probe was extracted with the Gene Clean II kit (Bio 101, Inc., La Jolla, CA). 5'-End labeling of probes was performed with T4 kinase (19). Probes were incubated with [γ-32P]ATP 10 μCi/ml (Amersham Corp., Arlington Heights, IL) in 25 mM Tris/HCl (pH 7.5), 10 mM MgCl2, 1 μl of β-mercaptoethanol (diluted 1:12.6), and 10 units of T4 kinase (Promega) in a 50-μl volume at 37°C for 60 min. Unincorporated 32P was separated from the probe, which served as a control for the amount of RNA loaded to each lane (19).

**Immunoprecipitation of ER Protein.** Cells were lysed in high salt lysis buffer [0.4 M KO, 20 mM HEPES (pH 7.4), 1 mM DTT, and 20% glycerol] supplemented with protease inhibitors (10 mg aprotinin/ml, 50 mg benzamidine/ml, 50 mg leupeptin/ml, and 40 mg phenylmethylsulfonyl fluoride/ml), followed by passing through a 26-gauge needle six times. After centrifugation at 100,000 x g for 20 min at 4°C, the supernatant (cytosol) was removed, and total protein was determined (Micro BCA protein assay reagent kit; Pierce, Rockford, IL). The cytosol fraction was incubated with the human ER monoclonal antibody H226 (12 μg/ml; Abbott) followed by the addition of 133 μg/ml protein A-Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ). The pellet was centrifuged and washed with PBS. The pellet was dissolved into loading buffer, and protein was separated on a 7.5% SDS-polyacrylamide gel for 2 h at 100 mV. The protein was then electrophoretically transferred to a nitrocellulose membrane and incubated with the monoclonal antibody to ER (1 μg/ml) for 2 h. Finally, the membrane was developed with the 3,3'-diaminobenzidine antibody kit to visualize the protein band. Controls included omission of the primary antibody (H226) and using MCF-7 and COS cells as positive and negative controls, respectively.

**Establishment of Tumor Xenografts.** SK-ChA-1 cells were grown in RPMI 1640 media supplemented with 10% heat-inactivated FCS in T75 flasks. Cells were trypsinized, washed with Dulbecco’s PBS (Cellgro), and counted with a Coulter counter (14). Mice were anesthetized with isoflurane inhalation, and 5 X 106 cells were inoculated s.c. into the flanks of mice using a 22-gauge needle in a total volume of 0.2 ml/site. Two weeks were allowed for tumor engraftment; the tumor engraftment rate was ~90%. Animals with similar tumor sizes were assigned to the tamoxifen or control group in a blinded fashion. The treatment was performed independently and in a blinded fashion. Drug therapy was initiated using 0.1 mg of tamoxifen injected i.p. three times per week or peanut oil with solvent alone in the control group. Caliper measurements of tumors were performed two times per week for 8 weeks in a blinded fashion as well (20). The mean tumor volume was calculated using the formula:

\[
\text{Tumor volume}_{\text{mean}} = \frac{1}{2} \times \left( \frac{\text{mean diameter}}{6} \right)^3
\]

The mean diameter is calculated from the average width and length of each xenograft in millimeters. Drug therapy and tumor measurements were performed over a period of 8 weeks, and no animals died during the study. The xenografts were resected aseptically, and tumor tissue was processed for routine histological examination by H&E staining and for immunohistochemistry to low molecular weight cytokeratin with a monoclonal antibody (AE-1; BioGenex, San Ramon, CA) as described (21).

**Statistical Analysis.** Data were analyzed using a nonparametric analysis with the Wilcoxon Rank Sum Test. P < 0.05 was considered statistically significant.

**RESULTS**

**In Vitro Growth Inhibition.** After allowing human cholangiocarcinoma cell lines to adhere overnight in culture, various concentrations of tamoxifen were added. At the indicated times, cells were either counted directly or indirectly by the colorimetric assay. Controls included either the addition of carrier alone (methanol) or no...
These results show that tamoxifen caused decreased proliferation of cholangiocarcinoma cell line, OZ, induced by tamoxifen. The various compounds tested included methanol, the carrier control, and graded doses of tamoxifen from 1 to 10 μM, a physiologically relevant dose. Data presented in a similar format with another cholangiocarcinoma cell line, SK-ChA-1, are shown in Fig. 2. Higher doses of tamoxifen (25 μM) were toxic. At the 5–10 μM concentrations of tamoxifen, there was significant and reproducible inhibition of tumor cell growth. The growth inhibition mediated by tamoxifen was not universal; specifically, the growth of HEP-G2 (human hepatoma cell line) and PANCl (human pancreatic cancer cell line) cell lines was not observed with similar concentrations of tamoxifen (results not shown). These results show that tamoxifen caused decreased proliferation of OZ and SK-ChA-1 cells.

**In Vivo Growth Inhibition.** For in vivo growth inhibition studies, SK-ChA-1 cells were chosen because this tumor line has been successfully grown as xenografts in athymic (nude) mice (16). Cells were inoculated onto the flanks of animals; 2 weeks were allowed for tumor engraftment. The tumor engraftment rate was ~90%. The growth of the SK-ChA-1 cholangiocarcinoma was determined by biweekly caliper measurements, and a significant increase in tumor volume was demonstrated in the control animal after 8 weeks. Tamoxifen was administered at a dose of 0.1 mg i.p. three times per week for a total of 8 weeks. Compared to control animals, tamoxifen caused a statistically significant growth inhibition in vivo of SK-ChA-1 xenografts grown in athymic (nude) mice (Fig. 3). There was no statistical difference in tumor size at day zero (initiation of drug treatment) between the two groups. However, the group of mice treated with tamoxifen for 8 weeks showed a significant reduction in tumor growth rate (P = 0.02) when using 0.05 as the significance level. This inhibition was observed in two separate experiments and performed with 21 mice bearing a total of 38 tumors. Importantly, this inhibition was achieved with a physiologically relevant dose of tamoxifen (22).

Microscopic evaluation of tumor xenografts with H&E staining revealed a moderately differentiated adenocarcinoma with biliary ductal formation and mitotic figures (Fig. 4A, ×62.5). Fig. 4B is a higher power view of the tumor (×125). An immunohistochemical stain with a monoclonal antibody to low molecular weight cytokeratins (AE-1) shows that the cells maintain their biliary phenotype (Fig. 4C).

**ER mRNA.** Molecular approaches were used to evaluate for the presence of ER mRNA in the cholangiocarcinoma cell lines. Fig. 5 shows an ethidium bromide-stained agarose gel following RT-PCR with RNA isolated from various cell lines. RT-PCR primers included ER primers on the left half of the gel and β-actin primers on the right half of the gel. Molecular weight standards are on the far right lane. Both human cholangiocarcinoma cell lines express ER transcript, as determined by RT-PCR. Equivalent amounts of β-actin were observed in the samples. MCF-7 (a human breast cancer cell line) and COS cells served as positive and negative controls, respectively. Although COS cells do not contain ER transcript, β-actin mRNA is present. Thus, by RT-PCR, ER mRNA is detectable in both human cholangiocarcinoma cell lines.

Northern blot analysis was performed to confirm the RT-PCR results and to determine the size of the ER transcript. ER variant mRNAs with deletions of portions of ER exons have been reported.
Thus, the presence of ER protein in human cholangiocarcinoma was confirmed by immunoprecipitation in both cholangiocarcinoma cell lines (Fig. 8). The H226 monoclonal antibody, which recognizes an epitope in the NH2 terminus of the ER, was used (24, 25). ER protein was identified in the positive control, MCF-7 cells, as well as both human cholangiocarcinoma cell lines, OZ and SK-ChA-1 cells. The COS cells did not contain ER protein. The Rainbow molecular weight markers are shown in the bottom lane. Thus, the presence of ER protein in human cholangiocarcinoma was confirmed by immunoprecipitation.

**DISCUSSION**

Cholangiocarcinoma is a malignant neoplasm of the biliary epithelium with devastating clinical consequences (1–5). The etiology of cholangiocarcinoma remains unknown; however, chronic inflammation of the biliary tract leads to an increased risk for development of cholangiocarcinoma. The disease is resistant to current systemic treatment including medical, chemotherapeutic, or radiation therapy. Surgery can be an effective cure; however, most patients present with unresectable disease or microscopically positive margins after surgery that eventually result in tumor recurrence via local tumor spread, peritoneal seeding, or direct extension into the liver parenchyma. Therefore, new treatment modalities are necessary to provide effective therapy for patients with metastatic as well as unresectable cholangiocarcinoma. Furthermore, the possibility of a prophylactic chemotherapeutic agent for those patients at increased risk for the development of biliary tumors, such as patients with PSC or congenital choledochal cysts, is an additional consideration (26). Tamoxifen may provide such new treatment.

Tamoxifen is a competitive inhibitor of estrogen binding to the ER. We have shown that tamoxifen inhibits the growth of two human cholangiocarcinoma tumor cell lines in vitro. Importantly, this growth inhibition was achieved with a physiological relevant dose of tamoxifen. Moreover, tamoxifen treatment resulted in a statistically significant growth inhibition of SK-ChA-1 human cholangiocarcinoma xenografts in nude mice. Finally, we have demonstrated the presence of ER at both the mRNA and protein level in two separate human cholangiocarcinoma cell lines. Thus, tamoxifen may have clinical utility and serve as an adjunctive chemotherapeutic agent, possibly improving the survival of patients diagnosed with unresectable or metastatic cholangiocarcinoma.

Whether the growth inhibition of cholangiocarcinoma occurs via the ER or another mechanism is unclear and will require further study.
Fig. 5. RT-PCR analysis for ER (left) and β-actin (right). RT-PCR demonstrated the presence of ER mRNA in both human cholangiocarcinoma cell lines (OZ and SK-ChA-1). Controls included MCF-7 human breast cancer cells (positive control) and COS cells (negative control). β-Actin was expressed by all cell lines and was used as an internal control for the presence of mRNA. Stds, molecular weight standards.

Fig. 6. Northern blot analysis for ER mRNA. Total RNA was isolated from MCF-7 breast cancer cells (Lane 1), both human cholangiocarcinoma cell lines (OZ (Lane 2), SK-ChA-1 (Lane 3)), and COS cells (Lane 4). Top. Northern blots were performed for ER, and full-length, 6.5-kb ER transcript was identified. Bottom, membranes were stripped and rehybridized with GAPDH cDNA probes.

Tumor growth inhibition by tamoxifen in ER-negative breast cancer has lead to studies on alternative mechanisms of the action of tamoxifen (27). Thus, alternative mechanisms include modulation of growth-inhibitory and -stimulatory factors (28, 29), induction of TGF-β (30—32), apoptotic mechanisms via Bcl-2 receptors (33, 34), tamoxifen binding to high-affinity microsomal binding proteins (35), effects of calcium influx (36), and inhibition of protein kinase C activity (37).

TGF-β has a plethora of biological functions important in the regulation of cellular proliferation and differentiation in most human epithelial cells (30). Investigations on the effects of tamoxifen on the growth of human breast cancer indicate that tamoxifen induces the autocrine secretion of TGF-β in human breast cancer cells, which then acts as an inhibitor of tumor growth (31). In addition, tamoxifen may induce TGF-β secretion by stromal cells leading to decreased cellular proliferation (32). Effects of tamoxifen on TGF-β levels in both the in vitro and in vivo experiments reported here have not been addressed but may provide additional insight into the mechanism of tamoxifen-mediated growth inhibition of human cholangiocarcinoma cells.

Prior to the establishment of these tumor cell lines of the biliary system, information on the biology of these cells was limited. The availability of these cell lines as well as new techniques for their isolation and long-term culture of bile duct cells has allowed a better understanding of various aspects of these cells (1). Other receptors on biliary epithelial cells include epidermal growth factor receptor (38), the secretin receptor (19), and the somatostatin receptor (39). Treatment of a cholangiocarcinoma cell line with a somatostatin analogue caused both in vivo and in vitro growth inhibition of tumor cell growth (39). Combination chemotherapy with somatostatin and tamoxifen...
chronic inflammatory diseases of the biliary tract such as PSC or congenital biliary cysts. Therefore, a prospective, randomized trial to evaluate the clinical relevance of the tumor growth inhibition of human cholangiocarcinoma mediated by tamoxifen demonstrated in this study is necessary.

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