Inhibition of Human Gastric Adenocarcinoma Xenograft Growth in Nude Mice by α-Difluoromethylornithine

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ABSTRACT

We studied the effect of inhibition of polyamine biosynthesis by α-difluoromethylornithine on the growth of a human gastric adenocarcinoma (CLEES) xenotransplanted in nude mice. CLEES is a well-differentiated gastric adenocarcinoma of the intestinal type. The doubling time has ranged from 7 to 10 days through 11 passages. Electron microscopic and immunohistochemical studies comparing the original tumor and xenotransplants showed similar structure and similar amounts of carcinoembryonic antigens. Polyamine biosynthesis is required for cell division. α-Difluoromethylornithine inhibits ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis. In this study, 48 athymic mice were used in two experiments. In the first experiment, two groups of 12 mice each were inoculated with CLEES tumors and received either tap water or a 3% α-difluoromethylornithine solution as drinking water. Tumor size was measured twice weekly. Tumor size was significantly decreased from controls by the fourth week of treatment and at all points of analysis thereafter for 7 wk. In the second experiment, α-difluoromethylornithine significantly reduced tumor concentrations of the polyamines putrescine and spermidine. In addition, the tumor content of DNA was significantly reduced in treated mice (0.64 ± 0.16 mg) compared to controls (4.76 ± 0.92 mg). Our data suggest that inhibition of polyamine biosynthesis may be a useful component of multidrug chemotherapy for human gastric adenocarcinoma. Establishment of tumor lines such as this gastric adenocarcinoma will facilitate further studies on the biological behavior of human gastric cancer and its response to chemotherapeutic manipulation in vivo.

INTRODUCTION

The incidence of gastric carcinoma has been declining in the United States since 1930; however, in 1987 there will be an estimated 24,600 new cases of gastric cancer, and an estimated 14,200 patients will die from gastric cancer (1). In some countries, notably Chile and Japan, gastric cancer is the leading cause of cancer death (1).

Patients with gastric adenocarcinoma have depended upon surgical resection for cure or prolongation of life; adjuvant therapy has not significantly altered the outcome of patients with gastric carcinoma after it has spread beyond the stomach. The development of techniques for xenotransplantation of malignant human tumors into athymic "nude" mice by Rygaard and Povlsen (2) has allowed reproducible human tumor transplantation into a living animal; in vivo responses of human tumors to therapy can thus be studied in detail.

The polyamines putrescine, spermidine, and spermine are essential for DNA synthesis, cell replication (3, 4), and the stabilization of DNA. DFMO* deprives cells of polyamines by inhibiting ornithine decarboxylase, the rate-limiting enzyme of polyamine biosynthesis. The inhibition of polyamine metabolism has recently been reviewed as it pertains to new therapies for cancer and for infectious diseases (4). Studies from our laboratory have recently shown that DFMO inhibits the growth of hamster (H2T) pancreatic adenocarcinoma (5) and mouse (MC-26) colon cancer (6) in vitro and in vivo and human gastric cancer in vitro (7, 8).

We report here the establishment and characterization of a human gastric adenocarcinoma cell line in nude mice and the inhibition of tumor growth by treatment with DFMO.

MATERIALS AND METHODS

Animals

BALB/c athymic mice, 4 wk old, were purchased from Life Sciences, Inc. (St. Petersburg, FL) and were used for the transplantation. The mice were maintained in a temperature-controlled isolation unit under specific pathogen-free conditions and were maintained with an ad libitum feeding schedule and 12-h light and dark cycles. All food, water, and bedding were sterilized.

Surgical Specimen Collection and Maintenance of the Tumor Line

The operative specimen of a human gastric adenocarcinoma was taken in a steroid manner, and a one-third portion was placed in RPMI 1640 tissue culture medium. Another one-third portion was minced, and then 3-mm³ fragments were implanted s.c. via an incision in the interscapular region of athymic mice (BALB/c). The final one-third was frozen in liquid nitrogen. Tumors were passed using sterile technique when they reached approximately 1 cm² in size. Tumors used for histological evaluation and for growth rate determination were not used for passage, but were allowed to grow to greater than 1 cm³ in size. To study tumor growth, we used interscapular s.c. implantation of 3-mm³ pieces of tumor. Tumor size (the longest perpendicular diameters) was measured by calipers twice weekly. Tumor area was expressed as the product of the two greatest perpendicular tumor diameters. Mean tumor area for the group of mice was calculated. Tumor doubling time (defined as the time in days required for the mean tumor area to double during the logarithmic phase of growth) was then determined. The size of the tumor was plotted on semilog paper, and the doubling time in days was calculated directly from the graph.

Hormone Receptor Analysis

Gastrin receptors were measured on cell membranes prepared from the tumor samples by the methods which we have developed and reported (9). Estradiol and progesterone receptors were measured in cytosol fractions of the tumor. Cytosol preparation and estradiol receptor measurement were carried out as published previously (10). Progesterone receptors were measured according to the method of Horwitz and McGuire (11).

Morphological Studies

Light Microscopy. Tissue blocks measuring 1 x 1 x 0.5 cm were fixed in 10% neutral buffered formalin for 6 to 8 h, processed routinely, and embedded in paraffin. Four-μm-thick sections were stained in hematoxylin and eosin and examined by light microscopy.

Electron Microscopy. Tissue blocks (1 mm³) were fixed in half-strength Karnovsky's fixative for 4 to 6 h, processed, and embedded in
Tumor doubling times were determined in passages 3 through 11. 85% of serially transplanted tumors in mice have grown. The tumor has been successfully transplanted in 11 subdesignated CLEES. The first passage was carried out after 29 gastric adenocarcinomas into nude mice. There has been tumor centrifuged at 10,000 x g for 10 min. Aliquots of the supernatant were analyzed using the avidin-biotin-peroxidase complex methods of Hsu and coworkers (12). The primary antibodies used were rabbit antihuman CEA (Dako Corporation, Santa Barbara, CA) and mouse monoclonal anti-CEA (Boehringer-Manheim Biochemicals, Indianapolis, IN). The second layer reagents were biotinylated goat antirabbit IgG (Fab'-); or biotinylated sheep antimouse IgG (Fab'-); (Cooper Biomedicals, Westchester, PA), and the third layer reagent was streptavidin-biotinylated peroxidase complex (Amersham Corporation, Arlington Heights, IL).

Details of polyamine determination and other methods are available upon request. The experiment was repeated once to assure reproducibility, and tumors were analyzed for polyamine levels at the termination of the second experiment.

Immuno-histochimical Studies

Sections (4 µm) of paraffin-embedded tissue blocks of the original and xenotransplants from athymic mice were examined for CEA, utilizing the avidin-biotin-peroxidase complex methods of Hsu and coworkers (12). The primary antibodies used were rabbit antihuman CEA (Dako Corporation, Santa Barbara, CA) and mouse monoclonal anti-CEA (Boehringer-Manheim Biochemicals, Indianapolis, IN). The second layer reagents were biotinylated goat antirabbit IgG (Fab')2; or biotinylated sheep antimouse IgG (Fab')2; (Cooper Biomedicals, Westchester, PA), and the third layer reagent was streptavidin-biotinylated peroxidase complex (Amersham Corporation, Arlington Heights, IL). Controls for the immunohistochimical studies included: (a) omission of primary antibody; (b) substitution of the primary antibody with preimmune serum of the same species; (c) omission of primary and secondary antibodies; and (d) screening of a panel of normal tissue controls and previously investigated and categorized tumor tissue samples, concurrently with the tumor under investigation.

RESULTS

Establishment and Characterization of CLEES

In 14 mo, attempts were made to transplant 10 separate gastric adenocarcinomas into nude mice. There has been tumor take in the nude mice with only one tumor, which we have designated CLEES. The first passage was carried out after 29 days. The tumor has been successfully transplanted in 11 subsequent passages with a mean time of passage of 30 days. In 2 yr, 85% of serially transplanted tumors in mice have grown. Tumor doubling times were determined in passages 3 through 11.

The tumor doubling time of passage 3 was 22.5 days, and the logarithmic growth phase began at approximately 19 to 20 days after inoculation. These mice received the tumor as a slurry as described above. The mean doubling time for passages 5 through 11 was 9 days with a range of 7 to 13 days. The latent phase before logarithmic growth begins has ranged from 9 to 20 days between passages. Once logarithmic growth occurred, however, the growth rates were similar.

Tumor samples from passage 7 were sent to the American Type Culture Collection (Rockville, MD) where isoenzyme characterization was performed by electrophoresis. These studies verified that human tissue was present in specimens removed from tumor-bearing mice and that this is not a spontaneously occurring mouse tumor.

Morphological Studies

Light Microscopy. Multiple sections of the CLEES xenotransplanted tumor revealed similar morphological features (Fig. 1). The tumor was composed of large and small glandular structures lined by stratified columnar cells with moderate cellular pleomorphism. Gland formation within glands was frequent. Large areas of necrosis within the tumor were present. The cells composing the glands were large and columnar with abundant cytoplasm with occasional vacuoles. The luminal borders were strongly eosinophilic. The nuclei were large and vesicular with dispersed chromatin and contained one to two prominent nucleoli. Nuclear pleomorphism was moderate, and mitotic activity was frequent. The overall picture was reminiscent of an intestinal type of gastric adenocarcinoma and resembled the original gastric tumor resected from the patient (Fig. 1).

Electron Microscopy. The tumor was a well-differentiated
DFMO INHIBITION OF STOMACH CANCER GROWTH

The tumor cells were tall and columnar with notable gland formation, and they had interdigitating adjacent cell membranes. The luminal aspect exhibited several well-formed tight junctions onto which bundles of tonofilaments converged. Well-organized microvilli decorated the apical borders. Moderate amounts of intermicrovillous vesicles were also present (Fig. 2). Intracellular pseudolumina decorated by microvilli were also frequently seen. The cytoplasmic organelles consisted of numerous mitochondria and free ribosomes with a few organized rough endoplasmic reticular profiles and prominent Golgi apparatus. Mucus granules were rare. Membrane-bound secretory granules were present subapically near the luminal surfaces.

Immunohistochemical Studies

Both the primary tumor and the transplanted tumor expressed CEA. The staining was appreciable, predominantly in the apical surfaces of cell-forming glands, while several other less-differentiated cells exhibited cytoplasmic staining (Fig. 3). Approximately 50% of the tumor cell population exhibited CEA immunoreactivity. The staining pattern was similar with both the polyclonal and monoclonal antisera, but the staining intensity with the monoclonal antibody was less, due to the nonprecipitating nature of the antibody.

The negative controls for the CEA immunohistochemical studies did not exhibit any nonspecific binding.

Hormone Receptors

Neither the primary tumor from the patient nor xenotransplanted tumors were found to have >1 fmol of specifically bound hormone/mg of protein for gastrin, estradiol, or progesterone.

Effects of DFMO on Tumor Growth

DFMO is remarkably well-tolerated. Ingestion of a 2 to 3% DFMO solution p.o. in mice results in ingestion of 3 to 5 g/kg/day of DFMO with only occasional diarrhea reported (15, 16). In our present study, mice receiving DFMO showed no signs of toxicity and maintained body weights similar to controls. There was 100% survival of mice in control and treatment groups in the relatively short experiments until termination at 7 wk.

In the first and second experiments, 2 mice in each group did not develop tumors, and these mice were excluded from study. Mice receiving DFMO showed no signs of toxicity. The control group gained more weight during the study, but differences in body weight could be attributed to differences in tumor weight (Fig. 4). In the first experiment, the tumor doubling time in the control group was 9 days, and logarithmic growth began on Day 22 after implantation. Tumor doubling time in the mice that received 3% DFMO drinking water was 15 days with logarithmic growth beginning on Day 32. The onset of logarithmic growth was delayed by 10 days, and the tumor doubling time was prolonged by 67% from controls.

Similar results were obtained in the second experiment (Fig. 5). The tumor doubling time was prolonged by 71%, which was 7 days in the controls and 12 days in the DFMO group. The logarithmic growth phase began on Day 14 in controls and Day 20 in the DFMO treatment group. By the fourth week of treatment, the area of the tumor was significantly smaller in the DFMO treatment group, and by the seventh week of treatment, the DFMO treatment group tumor area was less than one-quarter of the control (Fig. 5). DFMO treatment caused...
significant depletion of intracellular polyamine concentration and total DNA content in the tumors at the time of sacrifice. Tumor weights were 15% of control, and tumor DNA content was 13% of control. In addition, the concentration of DNA of the DFMO group, expressed as mg/g of wet weight, was only 80% of the control group (Table 1).

**DISCUSSION**

We have successfully established a human gastric adenocarcinoma in nude mice, which we have designated CLEES. Others have previously reported xenotransplantation of human gastric carcinoma into athymic mice (17–26). Tumor doubling time for the CLEES gastric adenocarcinoma is similar to reported doubling times in two studies (19, 23), which report doubling times from 5.6 to 12.1 days. The CLEES doubling time has varied from 7 to 13 days. Ezaki and coworkers (23) reported doubling times through 20 passages, which ranged from 5.6 to 11.2 days, with no trend of increasing or decreasing growth rate.

The low rates of success in the xenotransplantation of gastric carcinoma in our experience are also similar to the findings of others who have reported initial tumor takes of 1 of 6 Control, 2 of 9, and 3 of 13 (19).

Other investigators have reported that tumor xenografts in nude mice show no karyotypic shift and retain their original biological properties and structure (27), as well as antigentic properties (27) and the production of CEA (24). The CLEES tumor in passage 9 continued to express CEA in a fashion similar to the resected specimen from which it was established. We have demonstrated histological similarity of the xenotransplanted tumor to the original tumor. These properties make tumor xenografts useful models for examining human tumor biology and for testing anticancer agents (25, 28, 29).

Sumiyoshi and coworkers (26) have shown that pentagastrin promoted growth and increased the labeling index of a xenotransplanted human gastric adenocarcinoma (CS-6-JCK) and that cyclic AMP levels in the CS-6-JCK tumor were increased by a single i.p. injection of pentagastrin. They did not perform gastrin-receptor analysis.

We have not yet tested the effect of pentagastrin on growth of the CLEES tumor; however, we have found no specific gastrin-binding receptors on either the primary tumor or the xenografted tumor. In our laboratory, gastrin receptors have been detected in the mouse colon carcinoma line, MC-26 (9, 30, 31), and in the AGS tissue culture cell line of human gastric cancer plus some freshly resected human gastric cancers (32), as well as some human colonic adenocarcinomas (9).

The polyamines, putrescine, spermidine, and spermine, are ubiquitous in nature and are essential for cell growth and differentiation (3, 4). Polyamines play an essential role in the metabolism and function of nucleic acids (33); tumors, as well as other rapidly growing tissues, exhibit elevated rates of polyamine biosynthesis. Depletion of intracellular polyamines causes slowing and eventual cessation of cell growth (3, 4).

The polyamine biosynthetic pathway begins with the conversion of arginine to ornithine in the urea cycle. Ornithine is then converted to putrescine by ornithine decarboxylase, the rate-limiting enzyme in the polyamine biosynthetic pathway. Putrescine is subsequently converted to spermidine by spermidine synthetase, and spermidine is then converted to spermine by spermine synthetase.

DFMO, a derivative of pentanoic acid, is a structural analogue of ornithine and is an irreversible inhibitor of ornithine decarboxylase, which binds to the enzyme in place of ornithine (34).

DFMO significantly inhibits the growth of several animal tumors (5, 6, 15, 16, 35–37). Marx and coworkers (6), in our laboratory, demonstrated a 50% reduction in tumor size after 2 wk of treatment in established mouse (MC-26) colon adenocarcinoma as well as inhibition of the rate of colon cancer growth. Barranco and coworkers (7, 8) recently showed inhibition of AGS human gastric cancer clones by DFMO in vitro. In the present study, we have shown that DFMO produced an 80% reduction in tumor size by 7 wk in the CLEES gastric adenocarcinoma in vivo. These effects are probably mediated by intracellular depletion of polyamines by DFMO; however, we have not performed studies with polyamine replacement in this in vivo system.

Fujimoto and coworkers (38) reported on the effects of DFMO on a human gastric carcinoma, ST-2, grown in nude mice. In contrast to our study, they found no effect on tumor growth. DFMO was administered in a dose of 500 mg/kg twice daily for 7 days to mice with 100-mg tumors. Ingestion of 3% DFMO in drinking water p.o. resulted in ingestion of 4 to 5 g/kg/day, and the larger daily dose and longer treatment administered in our study may explain the different results.

DNA repair after damage induced by chemotherapeutic agents may depend on polyamines to stabilize the double-helical configuration. DFMO may be useful as an adjuvant to other chemotherapeutic agents. DFMO has been shown in vivo to potentiate the effects of 1,3-bis(2-chloroethyl)-1-nitrosourea (39), cyclophosphamide (40), interferon (37, 41), 9-β-D-arabinofuranosyladenosine (36), and methylylglyoxal bis(guanylhydrazone) (42). DFMO potentiates a number of other agents in vitro including 5-fluorouracil on a human colon carcinoma (43). Saydieri and coworkers (44) from our laboratory recently reported potentiation of the inhibitory effects of DFMO by cyclosporin A on the growth of a hamster pancreatic cancer. Recently Luk and coworkers (45) reported inhibition of a human small cell carcinoma growing in nude mice as well as increased survival of mice with a cyclic regimen of DFMO administered p.o. compared to continuous p.o. administration of DFMO. Median survival in the untreated group was 7.6 wk and in the cyclic regimen of DFMO was 54.3 wk which ap-
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We have shown that our studies have extended our knowledge of the antitumor effects of DFMO on human tumor growth. DFMO inhibits the growth of a human gastric cancer growing in nude mice when given as a single agent p.o. Our results suggest that DFMO may be an effective adjuvant treatment for human gastric adenocarcinoma. Further studies are needed to establish optimal drug combinations, dosage schedules, and sequences for maximal effective treatment of this neoplasm.

REFERENCES

3. J. S., and Nomura, T. Heterotransplantation of cultured human cancer cells and human tumor growth in nude mice, when given as a single agent p.o. Our results suggest that DFMO may be an effective adjuvant treatment for human gastric adenocarcinoma. Further studies are needed to establish optimal drug combinations, dosage schedules, and sequences for maximal effective treatment of this neoplasm.

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