Enhancement of Radioresponse of a Mouse Mammary Carcinoma to Combined Treatments with Hyperthermia and Radiosensitizer Misonidazole

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ABSTRACT

The regrowth delay of a transplanted syngeneic mouse mammary carcinoma designated MT2 was used to estimate the effects of three-fold combination treatments: X-irradiation; hyperthermia and radiosensitizer; and misonidazole (Ro-07-0582). The experiment entailed five groups of experimental mice: untreated control; X-rays alone; X-rays plus hyperthermia (42-43°C); X-rays plus misonidazole; and X-rays plus hyperthermia plus misonidazole. X-Ray treatments consisted of 4000 rads administered locally to the tumors in two equal fractions at a 48-hr interval. Misonidazole (0.67 mg per g body weight) was injected i.p. 30 min before exposure to X-irradiation. Hyperthermia was administered 10 min prior to and for 17 min during irradiation.

The regrowth delay factor of 3.9 was obtained by administering combined treatments of the three agents (X-rays plus hyperthermia plus misonidazole). The enhancing effect on a syngeneic mouse mammary adenocarcinoma by the modality of treatment herein described lends support to the usefulness of combining X-rays with other agents in the treatment of neoplasms.

INTRODUCTION

There is currently a strong interest in combining radiation with drugs for treatment of neoplasms. The need for using agents additional to radiation alone for effective therapy, particularly of solid tumors, stems from the heterogeneous architecture of these tumors. Solid tumors usually contain regions of closely adjacent tumor cells, remote from blood vessels, and these cells are therefore in hypoxic condition.

As far back as 1909, it was observed that restriction of blood circulation, limiting oxygen supply, rendered human skin radioresistant (37). A group of investigators in England, led by Gray (5, 18), undertook to develop methods for combining oxygen with ionizing radiation in the treatment of human and animal neoplasms. An oxygen enhancement ratio of about 3, relative to X-rays alone, was obtained in our laboratory when thin sections of 3 different mouse and rat tumors were diffused with oxygen in vitro during X-irradiation before grafting to syngeneic hosts (14). Whereas encouraging results were obtained from in vitro studies, no satisfactory results could be achieved from in vivo treatments (16). This was mainly due to the fact that solid tumors are poorly vascularized; therefore, no adequate supply of oxygen could be introduced in situ (even under increased barometric pressure) to render the hypoxic cells more radiosensitive. Hence, regardless of the fact that oxygen is thus far the most potent radiosensitizing agent for hypoxic cells, its usefulness is limited where radiotherapy of solid tumors is concerned. Exploratory studies were therefore undertaken to synthesize electron-affinic chemical compounds which would mimic oxygen (1). A number of such electron-affinic compounds were recently made available by Roche Products, Ltd. One of these compounds, designated misonidazole (Ro-07-0582), was shown to selectively affect hypoxic cells cultivated in vitro (11, 21, 22, 33, 45, 46) and tumor cells grown in animals (4, 8, 9, 24, 32, 36, 39-41, 43). Although this compound by itself is unable to destroy the tumor cells, it does enhance the therapeutic effects of ionizing radiation on several types of murine tumors by a factor of about 2 (4, 9, 32, 39, 43).

The adverse effects of hyperthermia on neoplasms were initially noted in a human patient who developed very high temperature caused by another disease, erysipelas (49). Since then, a number of similar reports have appeared in the literature. Experiments on animals were also performed, but the results from various laboratories were conflicting. This was mainly due to the use of heterologous host-tumor systems and to inadequate instrumentation for precise heat measurements. Comprehensive reviews on this subject are available (for example, see Refs. 23 and 44). The renewed interest in hyperthermic treatments for neoplasms stems from basic research performed in various laboratories under well-controlled conditions. Briefly, it has been found that cancer cells are susceptible to heat injury (2, 29-31) and that increased temperature enhances the effects of ionizing radiation on tissue cells cultivated in vitro (2, 10, 35) and on malignant tumors grown in vivo (7, 17, 19, 25, 28, 42, 44, 50); hyperthermia also potentiated the effects of misonidazole (3, 12, 43). The positive results that we obtained in this laboratory from the experiments with oxygen (14) and hyperthermia (17) encouraged us to test the possibility of enhancing therapy of tumors by combining the 3 agents, X-irradiation, hyperthermia, and misonidazole. When these studies began, we were unaware that similar experiments using these 3 agents were also being performed by Stone (43) on the C3H mouse tumor system in vivo and by Hofer et al. (26) using L1210 cells in vitro.

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2966  CANCER RESEARCH VOL. 39
MATERIALS AND METHODS

A syngeneic mammary adenocarcinoma designated MT2 was chosen for this initial experiment. This tumor had developed in a female X/Gf mouse by treatment with urethan and X-rays (15). The tumor is carried by serial passages in syngeneic X/Gf mice and grows equally well in mice of both sexes. Its latent period (time elapsing from implantation of tumor graft to the time of detection of a tumor nodule) is 10.1 ± 0.9 (S.E.) days. Its volume-doubling time is 2.2 ± 0.5 days. The tumor was in its 122nd passage of transfer at the start of the experiment. Its growth characteristics remained standardized during this period of transplantation.

Morphologically, sections of tumors stained with hematoxylin and eosin showed nests of closely adjacent epithelial tumor cells, which were surrounded by wide bundles of fibrous connective stroma. Within the nests of tumor cells, mitotic figures at various phases of division are usually seen in actively proliferating tumors. A microscopic field of a tumor section is seen in Fig. 1. The morphological characteristics are retained, as seen in microscopic sections of passaged tumors studied periodically. Its cell cycle parameters were determined by the autoradiographic method and tritiated thymidine labeling. These are: Tpo, 14 hr; Tn, 11.8 hr; Ta, 6.5 hr; and Tc, 32.3 hr. Concerning its ploidy, the number of chromosomes in metaphase cells ranged from 30 to 46, with 42 predominant. Detailed accounts of methodology, etc., of both determinations of cell cycle parameters and ploidy will be reported in a separate paper. The data herein mentioned serve only to characterize the MT2 tumor. In view of the compactness of adjacent tumor cells and the considerable amount of fibrous stroma, it was anticipated that many tumor cells would be in hypoxic condition. On this basis, the MT2 tumor was selected from 5 types of syngeneic tumors available in this laboratory for the initial experiment herein described. (A quantitative determination of hypoxic cells is in progress.)

X-Ray Irradiation. A General Electric 250-kV Maximar X-ray machine operating at 200 kVp and 15 ma was used. The X-ray beam was filtered through 0.5 mm Cu and 1.0 mm Al; half-value layer equaled 0.65 mm Cu. The dose rate averaged 113 rads/min (±5%) at the target distance of 31.5 cm from the X-ray source to the center of the tumors as measured in a sham model using a Victoreen ionization chamber. The tumor-bearing mouse was placed in a lead shield cylinder with a 2.5-cm internal diameter and a 0.6-cm-thick wall. The tumor and the surrounding skin were carefully elevated above a longitudinal 0.2-cm-wide opening in the lead wall and placed at the surface of the lead shield; the tumor was kept in place by adhesive tape, with care taken to avoid obstruction of blood supply. This lead device has been used in many previous experiments and has proved effective in protecting the normal tissues (13).

Heating Procedure. A Model CMD-5 microwave generator (Raytheon Co., Burlington, Mass.; frequency, 2450 MHz) utilizing the 100-watt Model C director served as the heating source. The director was placed 2 inches below a platform such that the microwaves were directed through a 1 x 2-inch opening. The lead shields were placed in such a way as to have only the tumors positioned over this opening. All additional areas, including the lead cylinders, were shielded by using a 20- x 20-inch copper mesh (0.016-inch wire thickness) to screen out extraneous radiofrequency waves.

Temperature measurements were taken using implantable thermistor probes connected to a Model BAT-8 continuous digital temperature readout (Bailey Instrument Co., Inc., Saddlebrook, N. J.). The probes were calibrated against a National Bureau of Standards Model 15-040A thermometer (Fisher Scientific Co., Pittsburgh, Pa.) by using a stable water bath at 3 different temperatures. The exposed metal shields on the thermistor probes were loosely wrapped with copper mesh to diminish the possibility of hot spots and erroneous temperature readings.

Probes were inserted from above into the core of the tumor of a reference mouse, and measurements were made simultaneously within the tumor and in the rectum. Body temperatures ranged between 37 and 38 ± 0.5°.

The intratumor temperature was brought up to 42–43° within 5 to 8 min before the start of treatment. This temperature was allowed to stabilize for 2 to 5 min, and then X-irradiation from above was added for the last 17 min to deliver 2000 rads to the tumor.

The monitoring and control of the temperature readout and microwave generator were maintained throughout the radiation treatment by use of an additional outside variable a.c. power transformer connected to the microwave generator (Dan Leoni, Raytheon Co.), and additional 6-ft extension leads were attached to the Model EXT-6 thermistor probes (Bailey Instrument Co.). Therefore, the intratumor temperature was constantly regulated to 42.5–43° during the radiation treatment time. Fig. 2 illustrates our X-ray and thermal equipment. Microwaves were proved to penetrate deep into the tissue (50).

Radiosensitizer. Misonidazole (Ro-07-0582) was obtained through the courtesy of Dr. W. E. Scott of Hoffmann-LaRoche Co., Nutley, N. J. It was dissolved in warm sterile 0.9% NaCl solution at a concentration of 30 mg/ml and was freshly prepared prior to injection into the experimental mice.

Experimental Design. Seventy X/Gf males about 4 months old were grafted between the axillary and inguinal region with about 2-mm MT2 tumor particles by the trocar technique. The MT2 grows in 100% of the grafted isogenic X/Gf mice. Of the 70 mice grafted, 60 with approximately 0.4-cu cm tumors were selected and divided into 5 groups as follows: Group 1, untreated controls; Group 2, X-rays plus hyperthermia (42.5° for 27 min); Group 3, X-rays plus misonidazole (0.67 mg/g body weight); Group 4, X-rays plus hyperthermia (42.5° for 27 min) plus misonidazole (0.67 mg/g body weight); and Group 5, X-rays alone. The X-ray treatments for all 4 groups consisted of 4000 rads delivered to the tumors at 48-hr intervals in 2 exposures of 2000 rads each. This dose was chosen as an initial step for future elaborate studies on time-dose relationships in combination therapy. The hyperthermic treatment lasted 27 min. This period included pre-X-ray and X-ray treatment. Misonidazole (0.67 mg/g body weight) was injected i.p. 30 min before X-ray treatment. The animals in the cages were inspected daily. Measurements of the tumors, control and experimental, were taken with vernier calipers 3 times weekly. The volume of tumors was calculated in cu mm by the formula:

\[ V = \left( \frac{e}{6} \right) \times D_1 \times D_2 \times D_3 \]

where \( D \) is diameter (mm).
RESULTS

Growth curves of the untreated control and of experimental tumors are illustrated in Chart 1. It is apparent that all the tumors of the 4 treated groups regressed at approximately the same rate for about 10 days, while the tumors of the untreated control mice grew progressively. Further, the time needed for the treated tumors to regrow to 50% of the original size was also taken as a criterion in evaluating the 3 modalities of treatment. Thus, it took 26 days for X-rays alone, 37 days for X-rays plus hyperthermia, and 53 days for X-rays plus misonidazole to reach 50% of the original tumor volume (0.4 cu cm). The tumors treated with all 3 agents continuously regressed after treatment, remaining at the lowest volume as compared with the tumors in the other treated groups and particularly with the tumors of the untreated controls.

Table 1 shows the ratios of tumor volumes at 54 days post-treatment of the 4 treated groups and of the untreated controls. Fifty-four days was chosen, since at this time some of the regrowing tumors were beginning to ulcerate. It is apparent that the tumors of those mice that were treated with all 3 agents remained at the lowest volume as compared with those tumors of the 3 other treated groups and particularly with those tumors of the untreated control mice. With respect to regrowth delay, it is also shown in Table 1 that the tumors treated with the 3-fold combination responded dramatically and resulted in the regrowth delay factor of 3.9 relative to X-irradiation alone.

The effectiveness of the combined treatments (X-rays plus hyperthermia plus misonidazole) is also expressed in the survival time of the treated mice (Table 2). The largest proportion of mice surviving were those treated with the 3-fold combination. At 120 days after treatment, 9 of the 11 mice alive were apparently tumor free. The proportion surviving in this group was significantly larger than in the group treated with X-rays alone (p < 0.01).

DISCUSSION

Enhancement of radiation effects by combined treatments with misonidazole has been reported by a number of investigators (4, 9, 32, 39, 43). Combined treatments of X-irradiation plus hyperthermia (7, 17, 19, 25, 30, 50) and combined treatments of hyperthermia plus misonidazole (3, 12, 43) have all been shown to enhance therapeutic results of solid tumors.

As recorded in Table 1, the combined treatment with the 3 agents resulted in a significant therapeutic enhancement for the mammary adenocarcinoma MT2, since 9 of 12 treated tumors completely regressed whereas no complete regression occurred among the mice treated with X-rays alone. Only a single tumor regressed in each group treated either with X-rays plus misonidazole or with X-rays plus hyperthermia.

Extensive experiments are in progress to test all 3 agents in single and multiple treatments. It is hoped that the results described in the present paper and those obtained from the current studies will be useful in the therapy of human tumors.

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Fig. 1. Microscopic field of a section of the mammary carcinoma designated MT2. Note nests of closely adjacent tumor cells (N), mitotic figures in metaphase (arrow), bundles of fibrous stroma (S), and blood vessel (BV). Other characteristics of the MT2 tumor are described in the text. H & E, × 350.

Fig. 2. X-ray machine and microwave (Thermo Apparatus). In a, the Raytheon microtherm unit with microwave director Model C is placed 2 inches below a plywood platform (P) which is covered by a 20- x 20-inch copper mesh (0.016-inch wire thickness) in such a manner that the microwave radiation is directed through a 1- x 2-inch cutout (arrow). The lead shield (LS) with the tumor elevated is placed above this cut-out. Only the tumor receives hyperthermia plus X-irradiation. M, Rice sham model. Two thermistor probes (TP) are inserted into opposite sides of the tumor. The tumors are irradiated at a distance of 31.5 cm from the X-ray source (X). b, control panel outside of the X-ray room. General Electric Maximar 250-III X-ray unit (X). Bailey digital temperature readout (T). A Variac a.c. power transformer (V) is connected to the microwave generator to monitor and control the temperature during X-ray treatments.
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