Tokai J Exp Clin Med., Vol. 9, No. 4, pp. 331–337, 1984

The Effect of Hypoxic Radiosensitizer after Mild Hyperthermia in C3H Mammary Carcinoma

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(Received October 9, 1984)

The radiosensitizing effect of misonidazole after hyperthermia was investigated in C3H mammary carcinoma. The tumors transplanted into the flanks of the mice were heated in a 42.3°C water bath for 30 min. When misonidazole was administrated before heating, the subsequent radiation effect was prominently enhanced, whereas postheating administration of misonidazole did not enhance the radiation effect significantly. The effect of varing the time between heating and radiation with or without misonidazole was as follows. Without misonidazole, the radiation effect was decreased at 6 hours after heating but increased at 12 hours, then it returned to the initial level at 24 hours and remained until 96 hours after heating. With misonidazole administered 30 min before irradiation, the radiosensitizing effect was observed at 24, 48 and 96 hours after heating. However, the total effects of this procedure were almost the same as the results in the combination without heating. Changes in the hypoxic fraction after hyperthermia are also discussed.

(Key Words: Hyperthermia, Misonidazole, Hypoxic cell fraction, Reoxygenation, C3H mammary carcinoma, Hypoxic cell radiosensitizer)

INTRODUCTION

It is known that hyperthermia enhances the effect of many drugs and radiation on mammalian cells (3, 4, 5, 12, 17). Recently, hyperthermia has been applied in the treatment of cancer together with radiotherapy and chemotherapy (8, 10, 11, 15, 18). In these cases, one of the most important problems is the time and order of application of drugs or radiation and hyperthermia to obtain maximum effectiveness against tumors.

Hyperthermia alters the blood flow in most tumors (1, 17). Song et al. (16) reported that 43.5°C for 30 min decreased blood flow and increased the hypoxic fraction in SCK mammary tumors at 5 to 24 hours after heating. It is suspected that the changes in blood flow caused by hperthermia have an influence on the effect of drugs or radiation.

Misonidazole is known as a hypoxic cell radiosensitizer and is considered to reach hypoxic cells situated far from capillaries (2, 6). Although 5 mM of misonidazole is cytotoxic for hypoxic cells, it has neither a cytotoxic nor a radiosensitizing effect on aerobic cells in vitro (7, 14, 20). The radiosensitizing effect of misonidazole has been potentiated by concomitant use with hyperthermia (9, 19, 21). However, the effect of misonidazole after heating is not known.

The above effect was studied and the most effective time of radiation was determined after heating using the growth delay assay in C3H mammary tumors. Heating at 42°C for 30 min was chosen, taking into consideration the easy applicability of clinical hyperthermia.

METHODS AND MATERIALS

Animals and tumors: Male C3H/He mice obtained from Takasugi Inc. were used. They were fed under normal conditions and used in the experiment at 6–8 weeks of age. FM3A mammary carcinoma that arose spontaneously in C3H/He mice was adapted for in vitro growth and was maintained according to con-

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ventional techniques. Two to 6×10^6 cells suspended in saline were injected subcutaneously into the flanks of the mice. After 4–10 days the tumors became palpable, and their three-dimensional diameters were measured 3–4 times per week.

Heating of tumors: When the tumors grew to 8–9mm in mean diameter, heating procedures were carried out with a water bath. The mice were not anesthetized through this study. They were immobilized with tape on a styrofoam board. The right leg and the flank with the tumor were passed through holes in the board and the leg was fixed with tape. The tumor was pulled vertically by the sinker tied with thread to the skin near the tumor. When the temperature of the water bath was kept at 42.2–42.4°C, the tumor was heated to 42.0–42.2°C within 3 min. The tumor temperature was monitored during heating with thermocouple microprobes (Bailey Instruments, Inc., Saddle Brook NJ.). Body temperature as measured rectally was raised about 1°C. Using an electric fan, steam was blown over the surface of the water during heating. Total heating time was 33 min. Misonidazole was injected peritoneally at various time intervals after heating.

Misonidazole: 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol was provided by Roche Products, Ltd. It was dissolved in 0.9 per cent NaCl to give 20mg per mℓ. One mg of misonidazole per gram of body weight was administered intraperitoneally 30 min before irradiation.

Irradiation: The mice with tumors injected or not injected with misonidazole were irradiated with Co-60 teletherapy units (Theratron 780, Canada) at 0.5, 6, 12, 24, 48, or 96 hours after heating. They were immobilized with tape on 5-mm aklyl board, and the tumors were pulled from the abdominal wall gently with the tape. The tumors were irradiated through the board at doses of 10, 20 or 30 Gy. Dose rate was 2.33 to 2.04 Gy/min at 45cm of source-skin distance, and the field size of irradiation was $4.5 \times 4.5 cm^2$.

Assay: The effects of various treatments were determined by the duration of growth from 7 to 11mm in mean diameter (Fig. 1). An increase in diameter from 8mm (on the treatment day) to 11mm meant an almost twofold increase in volume. Few tumors regrew to 11mm in diameter after 60 days, and these were not included in the growth delay assay. The mice with cured tumors at 60 days after treatment survived without recurrence until sacrifice at 120 days.

Treatment designs: (1) Radiation dose-growth time in relation to hyperthermia and irradiation with or without misonidazole. When misonidazole was used, it was administered immediately after heating, and varying doses of irradiation were given at 30 min after heating. 2) Influence of the sequence of misonidazole application. Twenty Gy of irradiation was given within 30 min after heating. Misonidazole was administered immediately before or after heating. 3) The effect of irradiation with or without misonidazole at varying times after heating.

In total, six experiments were carried out in each group with almost the same composition, especially in the comparative experiments. Six to nine animals were used in each group.

RESULTS

Fig. 1 shows typical examples of various growth curves: untreated, irradiated with 20 Gy, head at 42°C for 30 min with or without 20 Gy of irradiation, and combined heating, irradiation and 1.0 mg/g misonidazole administered either immediately before or after heating. Although heat alone did not greatly affect growth curves, the radiation effect was enhanced by the combination with heating. Preheating administration of misonidazole potentiated the combined effect of heat and radiation.

Fig. 2 shows the dose versus growth time curves (the time nesseary to grow from 7 to 11mm in mean diameter) for irradiation alone and for irradiation combined with misonidazole and/or hyperthermia. In the case of irradiation alone, the curve shows a straight line with a mild shoulder at low doses of radiation. When hyperhermia was followed by irradiation, the curve became steeper than that of irradiation alone. Hyperthermia alone prolonged growth time to about two days (Table 1). These results indicate that hyperthermia sensitizes the radiation effect. When misonidazole was administered immediately after heating and before irradiation, the curve showed little dif-

ference from the curve for the combination of hyperthermia and radiation without misonidazole. Also, its combination did not exceed the effectiveness of the combination of radiation and misonidazole without hyperthermia (Fig. 2). However, when misonidazole was administered immediately before heating and radiation, a remarkable effect was observed. This differed significantly from the effects of the administration of misonidazole after heating, as shown in Table 1 ($p < 0.01$). These results indicate that misonidazole might lose its effectiveness if administered after heating.

Fig. 3 shows the changes in the effect of irradiation with or without misonidazole at 12 to 96 hours after heating. The observed data varied greatly with each group (Fig. 3), but these curves were drawn by the least square method. The dashed lines represent the dose-response curves by iradition at 0.5 hour after heating. The curve for the 12-hour interval between heating and irradiation was steeper than that of the 0.5-hour curve, but the dose-effect curves at 24 to 96 hours after heating were almost the same as that of the 0.5-hour curve. In Fig. 4, the various time-interval results are illustrated. These were calculated from the data in Fig. 3. The radiation effect was minimized at 6 hours and increased at 12 hours after heating. From 24 to 96 hours after heating, the effect was decreased again and became almost the same as the effect at 0.5 hour after heating. Compared with radiation alone, potentiation by hyperthermia was observed even after a 96-hour interval. The combined effect of radiation and misonidazole was obtained after more than a 12-hour interval, and the effect was almost equal to the values obtained from the combination of the same dose of radiation and misonidazole without heating. When misonidazole was added in addition to the combination of heat and radiation, the effect of misonodazole was less prominent at 0.5 and 12 hours after heating, but became obvious from 24 to 96 hours after heating.

DISCUSSION

It is well known that hyperthermia enhances the effect of radiation in vitro and in vivo. The degree of enhancement depends on the sequence of their combination (3, 4, 16). In general, the effect decreases when the interval betweeen irradiation and heating is long. One reason may be a decay of the potentiation of the radiation effect by heat. Another reason may be an increase in hypoxic cells induced by hyperthermia. Song et al. (16) reported an effect on the proportion of hypoxic cells in SCK mammary tumors by heating at 43°C for 30 min. The hypoxic fraction increased at 5 hours after heating and decreased thereafter. The increase in the hypoxic fraction may be attributed mainly to vascular occulusion, and the diminution in the hypoxic fraction from 5 hours after heating was accounted for by an increase in the oxic cell number. They analyzed the hypoxic fraction in tumors using radiation survival curves of aerobic and hypoxic tumors. We estimated the hypoxic fraction using the hypoxic cell radiosensitizer misonidazole. The administration of 1mg/g misonidazole is estimated to produce about 0.5 to 0.75 mM of misonidazole concentration in this tumor (13). Aerobic cells are not affected at this concentration in vitro. From the enhancement of misonidazole, the fraction of hypoxic cells could be estimated, assuming that misonidazole was always fully incorporated in the hypoxic cells and the interaction between misonidazole and hyperthermia was negligible.

However, it is known that the effect of misonidazole is also sensitized by hyperthermia (9, 19, 21). When misonidazole was administered before hyperthermia, the effect on tumor response was prominently enhanced. But the enhancement of misonidazole immediately after hyperthermia is lower that of misonidazole without hyperthermia (Fig. 2). This may be accounted for by the fact that hyperthermia after the administration of misonidazole enhances the uptake of misonidazole into cells due to increased blood flow, while hyperthermia before misonidazole inhibits the uptake into the cells as a result of decreased blood flow by hyperthermia. It is difficult to estimate the hypoxic fraction using the hypoxic radiosensitizing effect of misonidazole when used with short intervals of hyperthermia.

Changes in blood flow produced by hyperthermia have been reported in many tumors (1, 17). Hyperthermia reduces blood flow in most tumors. Bicher and Vaupel (1), using an ultramicroprobe, reported that hyperhermia over 41°C decreased oxygen concentration in

C3H mouse mammary carcinoma. Stewart and Begg (17) studied changes in perfusion after 42.5°C for 1hr using the ^{86}Rb extraction technique. They observed a reduction low for 1–2 days before returning to control values. Hasegawa et al. (to be published) have observed changes in blood flow at 42°C in CH3 mammary carcinoma. Decreased blood flow immediately after hyperthermia returned to its initial level after 12 hours. In our study, when radiation was given after heating, the effect decreased at 6, 24, 48, and 96 hours and increased at 12 hours. However, when misonidazole was added before irradiation, the sensitizing effect of misonidazole was prominent from 24 to 96 hours, but the effect at 12 hours was almost the same whether misonidazole was present or not. Therefore, even if misonidazole were taken in the hypoxic cells after 12 hours, increments in radiation effect at this time would be due to reoxygenation. After 24 hours, it is considered that misonidazole is taken into hypoxic cells because of the high enhancement effect by misonidazole, and it is suggested that this effect represents the misonidazole-sensitized hypoxic cell fraction. The hypoxic fraction from 24 to 96 hours after heating would be less than that in untreated tumors if hypoxic cells in heated tumors had the same enhancement ratio from misonidazole as that in unheated tumors. These events may be accounted for as follows. Although hyperthermia might kill hypoxic cells, occlusion of the blood vessels in a tumor would produce another hypoxic cell fraction at 6 hours after heating. Then reoxygenation might occur due to repair of the blood the flow or death of cells after 12 hours, and hypoxic cells might repopulate from 24 hours after heating.

Estimation of the hypoxic fraction by using a hypoxic radiosensitizer is inaccurate because of the influence of hyperthermia, especially at short intervals. Changes in the concentration of misonidazole in tumors have been studied using a polarographic technique (13). The presence of radio-resistant cells at 24 hours after heating leads to the conclusion that there are many hypoxic cells, and the combined use of radiation with misondazole might kill these cells, which are induced by heating. However, the effect is almost the same as that obtained by radiation and treatment of misonidazole without hyperthermia. Also, the effect of misonidazole at 12 hours after heating was little, perhaps because of decreased blood flow by hyperthermia. Thus when hyperthermia is given beforehand, we should consider the sequence of the following radiation and misonidazole treatments. Though misonidazole administered immediately before heating led to a prominent sensitizing effect on the tumor, hyperthermia may enhance the normal tissue response. Further investigation to resolve this problem is necessary.

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Table 1 Response of 20 Gy irradiation for C3H mammary carcinoma treated at various sequences with misonidazole and/or hyperthermia

Treatment	Duration to regrow to 11mm ± s.e. in days
Untreated	5.2 ± 0.6
Hy alone	7.1 ± 2.1
20Gy alone	13.7 ± 2.3
Mis-20Gy	30.4 ± 4.5
Hy-20Gy	24.9 ± 10.8
Ht-Mis-20Gy	25.5 ± 6.3
Mis-Hy-20Gy	45.6 ± 12.3

Hy : 42°C/30min.
Mis : 1.0mg Misonidazole

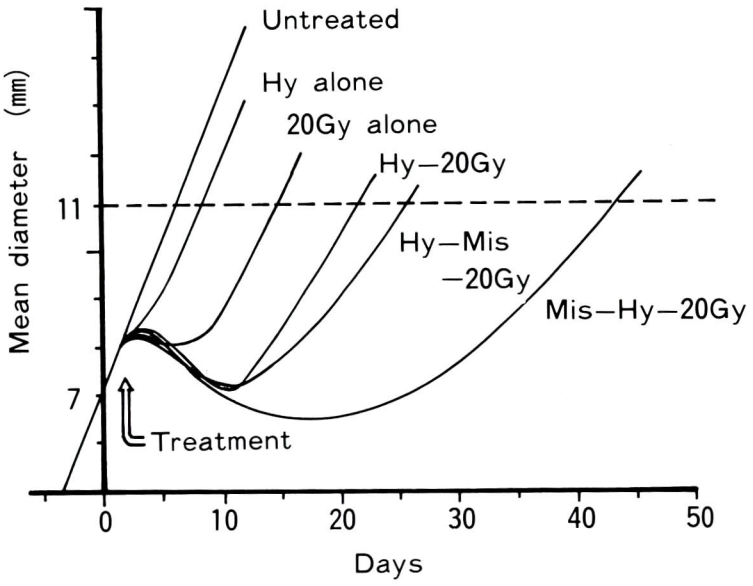

Fig. 1 Typical growth curves for C3H mammary carcinoma either untreated (control) or treated with 20 Gy irradiation, 42°C/30 min hyperthermia with or without 20 Gy and/or 1.0 mg/g misonidazole administered either immediately before or after hyperthermia. Each treatment was carried out in tumors of 8mm in mean diameter. The days needed for regrowth from 7 to 11mm in mean diameter were used to measure the effectiveness of experiments throughout this study.

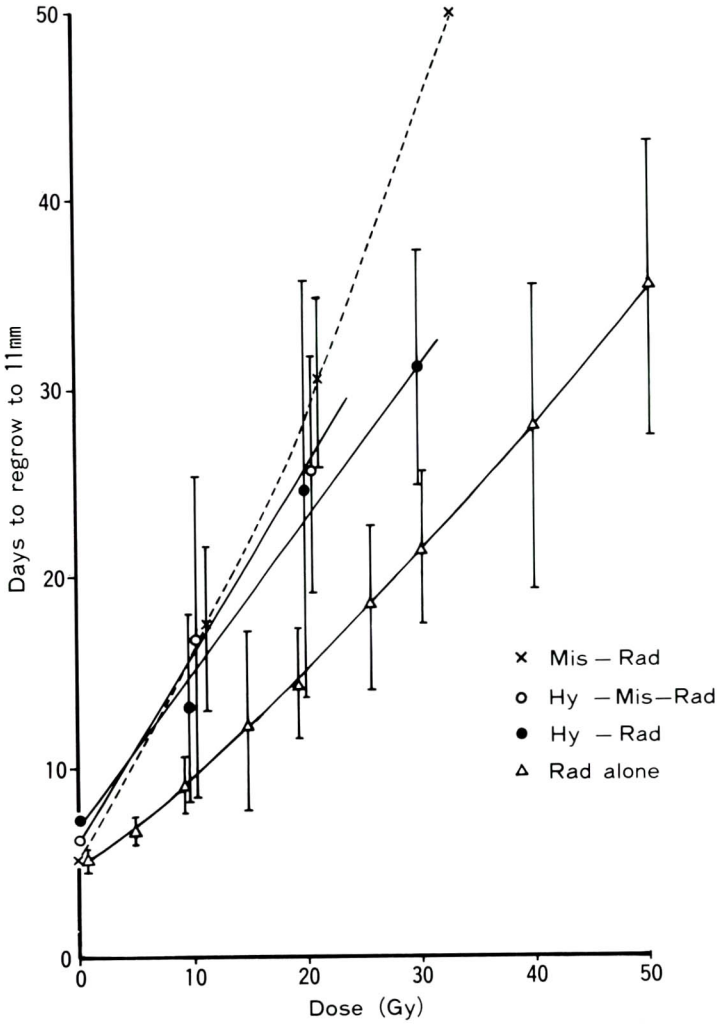

Fig. 2 Dose-response curves for control (△), misonidazole 30 min before irradiation (×), hyperthermia 30 min before irradiation (•), and hyperthermia with misonidazole at 30 min before irradiation (○). Each point represents the mean value and vertical bars represent standard error. (6–9 mice).

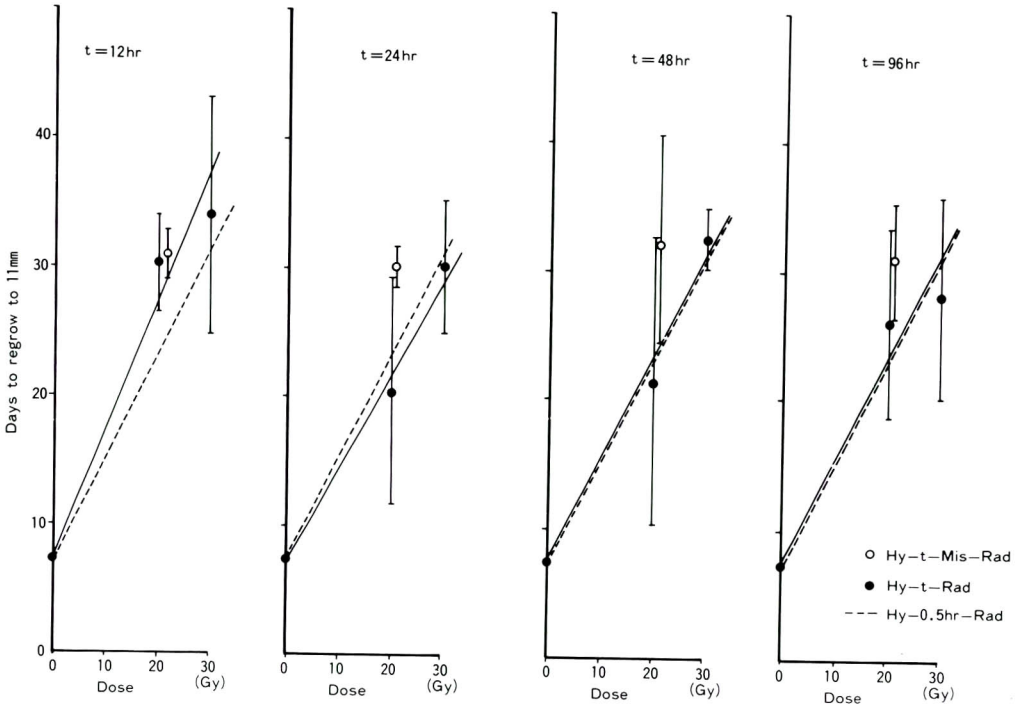

Fig. 3 Dose-response curves for radiation with (○) or without misonidazole (•) at 12, 24, 48, and 96 hours after heating. The solid lines which are drawn by the least square method represent the dose-response curves by radiation without misonidazole at each hour after heating. The dashed lines represent the dose-response curves by radiation with misonidazole at 0.5 hours after heating.

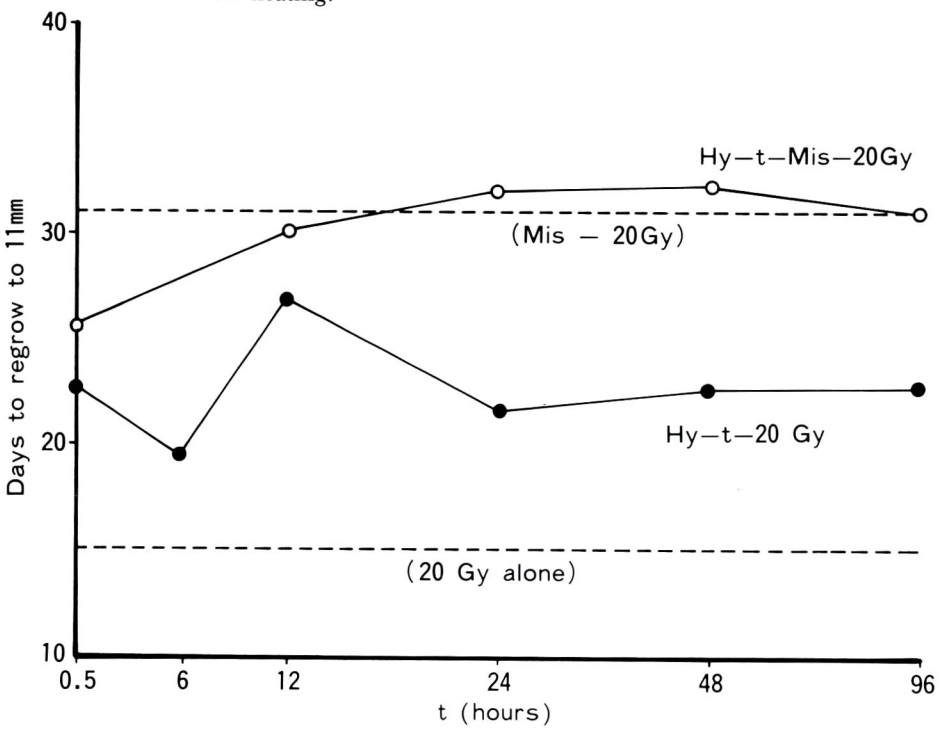

Fig. 4 Changes in response to 20 Gy of radiation with or without misonidazole as a function of time between heating and irradiation. The dashed lines represent 20 Gy of irradiation with or without misonidazole.