Radiosensitizing Effect of Combined Radiotherapy, Hyperthermia and Misonidazole on C3H Mouse FM3A tumor

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C3H マウス FM3A 腫瘍に対する放射線，ハイパーサーミアおよびミソニダゾールの併用効果

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C3H マウス FM3A 腫瘍に対する放射線，ハイパーサーミア（43℃，10分）およびミソニダゾール（0.5mg/g）の併用効果を調べた．

腫瘍（直径8mm）に対し，X線照射と加熱を同時に行うため，赤外線装置を作製した．加熱開始後，腫瘍内温度は2分以内に43℃に達し，以後温度を43±1/4℃に保つことができた．この装置により，X線照射と同時に，再現性良く腫瘍を加熱することが可能であった．

ハイパーサーミア(43℃，10分)とミソニダゾール（0.5mg/g）の単独または併用の場合，生長曲線で腫瘍の生長を抑制する傾向がみられたが，対照群と有意の差は認められなかった．半数の動物で腫瘍が実験開始時の3倍の容積に達する日数を求めたところ，ハイパーサーミア（43℃，10分）とミソニダゾール（0.5mg/g）併用群のみが対照群と比較し，有意に大きい値を示した．

TCD 50法を用いてX線との併用効果を調べたところ，TCD 50値として，X線単独で6024rad (1 s.d.＝268rad)，X線とハイパーサーミア（43℃，10分）の併用で5108rad (1 s.d.＝521rad)，X線とミソニダゾール（0.5mg/g）の併用で3,856rad (1 s. d.＝235rad)，X線とハイパーサーミア（43℃，10分）およびミソニダゾール（0.5mg/g）の三者併用で3,093rad (1 s.d.＝319rad)の値を得た．放射線増感比はそれぞれ1.0，1.2，1.6および1.9であった．この結果より，ハイパーサーミア(43℃，10分）とミソニダゾール（0.5mg/g）は放射線に対してそれぞれ独立して増感作用があると考えられた．

Introduction

The effectiveness of radiation therapy is increased when combined with the appropriate use of hyperthermia. Treatment of tumors by hyperthermia alone at temperatures of 41.5—45°C has been shown to produce local control[1)~3)]. Hyperthermia has also been shown to enhance the local tumor control effect of radiation[2),4)~6)].

The degree of the thermal sensitization depends on the sequence and separation of the heat and X-irradiation[7][8], therefore simultaneous administration of heat and X-irradiation is desirable. We designed an infrared apparatus, examined the stability of the apparatus and uniformity of the temperature of the tumor, and evaluated the possibility of simultaneous administration of X-irradiation and hyperthermia.

Misonidazole, 1-(2-nitroimidazole-1-yl)-3-methoxy-2-propanol (Ro-07-0582, Roche Products Ltd.), has been developed as a hypoxic cell sensitizer. Misonidazole selectively sensitizes hypoxic cells[9][10], and the doses of radiation have been shown to be reduced to about half[11]~[14]. Some reports have shown that hyperthermia enhances the cytotoxicity of misonidazole[14]~[18].

The purpose of the present investigation is to evaluate the possibility of the simultaneous administration of X-irradiation and hyperthermia using infrared apparatus, and to determine the effect of the X-irradiation, misonidazole and hyperthermia on local control of C3H mouse FM3A tumors using the TCD50 method[19].

Materials and methods

1. Tumor system

C3H/He mice of both sexes were supplied by Funabashi Farm Co., Chiba. They were kept in small animal units and provided with Purina pellets and water ad libitum. Ten to fourteen week old C3H/He mice were used. FM3A cells used in this study were derived from spontaneous C3H mouse mammary carcinoma, and maintained continuously as a suspension culture[20]~[22]. Cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum and 60 μg/ml Kanamycin, in 10 cm glass petri dishes at 37°C in atomosphere of 5% CO_2 in air. Cells were stored in liquid nitrogen and transplanted into the subcutaneous tissue in the thighs of mice as needed. Tumor cells were serially transplanted and animals carrying eighth generation tumors were sacrificed by cervical dislocation and the tumors were excised. Intact tumor tissue was finely minced with scissors and suspended in Hank's medium containing 5% fetal calf serum. The mice was sedimented for 15 minutes in iced test tubes. The supernatant fluid was removed by syringe and passed through a swinny filter and then centrifuged for five minutes. The sediment was resuspended with a small amount of Hank's medium and used for transplantation. Viable tumor cells were counted with a hemocytometer using 0.05% trypan blue, where dead cells were determined by trypan blue staining method, and a solution of $1.4 \times 10^5/\mu$l viable cells was obtained. Viable cells, 1.4×10^6 (10 μl), were transplanted subcutaneously into the right thigh. When the experimental tumors reached about 268 mm^3 (diameter $8 \times 8 \times 8$ mm), the mice were assigned a random number.

2. X-irradiation

Tumors were irradiated with single doses using a Toshiba KXC-18 type X-ray irradiation apparatus, operated at 180 KVp, 25 mA, with a 2 mm aluminum filter with an HVL of 8 mmAl, a focus-tumor distance of about 22.5 cm, and a dose rate of about 470 rad per minute to a field 2×2 cm in size. The mice were breathing normal air and were anaesthetized with pentobarbital administrated intrapritoneally. As the dose rate was about 470 rad per minute, the duration of irradiation reached about 12 minutes for 5500 rad. The duration of heating was 10 minutes. In low doses, the irradiation started 2 minutes after heating was started. In large doses of irradiation, irradiation and heating were started simultaneously.

3. Misonidazole

The solution of misonidazole (MISO) was prepared by dissolving the MISO in the solution of 0.9% NaCl to a concentration of 10 mg per ml and the doses of 0.5 mg/g mouse weight were administrated intraperitoneally 30 minutes before the irradiation.

4. The methods of local hyperthermia

Infrared apparatus was used for the local hyperthermia, where a new type of infrared ray source (BL-35, lamp type, Kokusai Denko Co. Ltd., Japan) was employed. The wave length of this infrared ray source is over 3.5 μ which is much longer than that commonly obtained from a infrared lamp (0.7—2.0 μ). This source is

stable, has a large thermal capacity (AC 100 V, 350 WV, the effective infrared ray radiation is 168 W), and a long life of over 16000 hours (a common infrared lamp, 4000-5000 hours). We added a shutter mechanism to this apparatus to obtain a delicate temperature control for the tumor because with this source it was difficult to make rapid changes in the intensity of infrared ray.

Fig. 1 illustrates a cross section of this apparatus. This apparatus includes two feedback systems. In the upper half of the apparatus, infrared ray is generated by the source and regulated by the feedback system, in which the thermistor (Th 1 in the figure) is used to keep the temperature roughly at 50-60°C in the upper section of the apparatus. The temperature is further controlled closely to 43°C with a shutter mechanism connected to two other thermistors in the lower section which delicately regulates the intensity of the infrared rays. The thigh of the mouse is fixed in the lower section. Thermistors are indicated as solid dots in the figure. One of the two thermistors is in contact with the tumor and the other is suspended in the air near the tumor. The latter thermistor is used for protection from overheating of the tumor (correcting for the heat capacity of the tumor). The inner surface of this apparatus is covered with foliated aluminum which reflects infrared rays. The body of the apparatus is made of styrofoam. This apparatus has a light weighed simple structure and is easy to assemble. The shutter is controlled with a digital proportional amplifier connected to

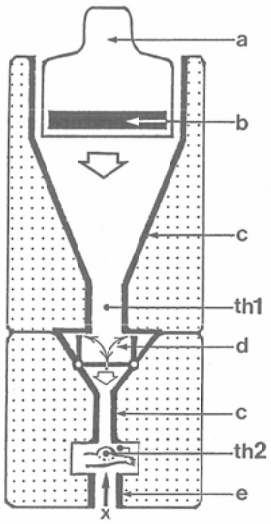

Fig. 1 A schematic representation of the ifrared ray apparatus. The infrared ray lamp is shown in (a). Infrared ray is generated from a flat discoid source (b). The inner surface of this apparatus is covered with foliated aluminum (c). A shutter mechanism (d) regulates the intensity of the infrared ray. The tumor is fixed in the lower portion of the apparatus. A thermistor (th 1) detects the temperature in order to control the output of the infrared ray. Two other thermistors and the tumor are located as shown (th 2). One thermistor is in contact with the tumor and the other is very near the tumor suspended in the air. The tumor is heated by infrared ray from above and irradiated from below (x). The surface area of the channel through which the X-rays pass is covered with lead (e).

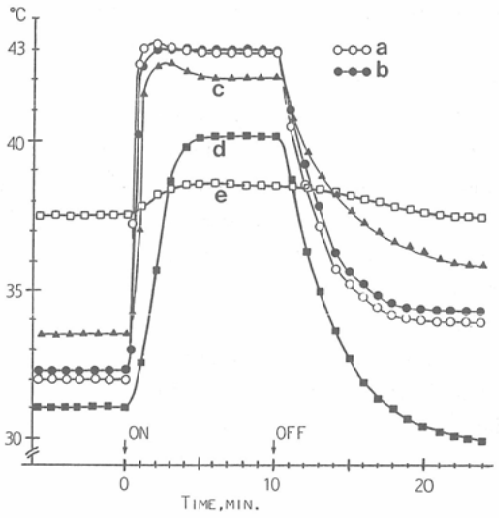

Fig. 2 The temperature of the tumor and other sites. These sites were measured simultaneously. The vertical axis shows the temperature measured (°C). The transverse axis shows the time, where time zero means the starting point of heating (ON). The heating was stopped (OFF) at ten minutes. The circles indicate the temperature of the surface of the skin (a) and the solid dots indicate the tempearture of the center of the tumor (b). The other temperatures are shown as follows —the foot pad of the heated site of the leg (c), the lower aspect of the thigh (d), and the rectum of the mouse (e).

the thermistor number 2. At the desired temperature, 43°C, open and close commands appear simultaneously in the circuit and these commands, acting competitively each other, stop the movement of the shutter and maintain it in its last position. This system reduces malfunctions and increases stability of the apparatus. The tumor illustrated can be irradiated locally simultaneously with hyperthermia. The lowest opening of the reflecting tube (C in the Fig. 1) just above the tumor is 1.5 × 6 cm in size (heating field) and the long axis crosses the thigh of the mouse.

Results

1. Local hyperthermia using infrared apparatus and simultaneous administration of X-irradiation and hyperthermia

Fig. 2 shows the temperature of the tumor (8 mm in diameter) and other sites measured simultaneously with multi-channel equipment. The temperature of the center of the tumor was measured with a needle type thermistor, and other sites were measured with small bead type thermistors. As shown in Fig. 2, the temperature of the surface of the tumor increased more rapidly than that of the center of the tumor. In about two minutes the temperature of the center and the surface of the tumor reached 43°C and was kept at 43 ± 1/4°C. The duration of heating was ten minutes. The rectal temperature rose about 1°C in this period and the temperature of the inferior aspect of the thigh rose to 40°C.

The temperature of the inner surface of the tumor (near the muscle of the thigh) also rose to 43°C within 2 minutes and was kept 43 ± 1/4°C thereafter.

We were successful in heating the tumor uniformly and in keeping the temperature constant. These results were reproducible when the mean size of the tumor was 6-10 mm in diameter, but if the mean size of tumor was over 10 mm or under 6 mm in diameter the results were unstable.

The tumor was irradiated through the thigh of the mouse (X in the Fig. 1). There was no influence on the infrared apparatus caused by the X-irradiation, and the simultaneous administration of heating and X-irradiation was possible.

2. Average tumor growth curve

Tumors were used when the mean diameter reached to 8 mm. After treatment, tumors were measured in size daily with vernier calipers. Volume was calculated by the formula $V=\pi(d_1)(d_2)(d_3)/6$, where d is the tumor diameter. The average tumor volume was calculated for each experimental assay group. Then, the relative volume was calculated using the average tumor volume at treatment as the standard.

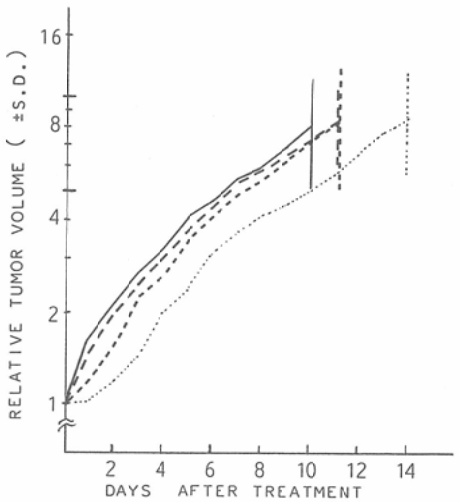

Fig. 3 Growth of C3H-FM3A tumors in control, misonisazole-treated mice (0.5 mg/g body weight, i.p.), hyperthermia-treated mice (43°C, 10 min), and mice treated with both misonidazole and hyperthermia. There were 7-13 mice in each group.
——— control; ——— misonidazole; -------- hyperthermia; ..... misonidazole + hyperthermia.

Growth curves of unirradiated tumors in the control group and those treated with misonidazole, or 43.0°C hyperthermia, or both, are shown in Fig. 3. The mean volumes of tumors reached the value of eight times the standard value (day=0) on the 10th day (control), 11th day (misonidazole or hyperthermia), and 14th day (misonidazole with hyperthermia). Although misonidazole or hyperthermia appeared to have a slight effect, there was considerable variation between individual tumors in the groups as reflected by the wide standard deviation. Therefore there was no significant difference statistically between the four curves. In the present experiment, no tumor control was found without X-irradiation.

3. Tumor growth time (X3)

Tumor growth time (X3) —days required for the tumors of 50% of the animals to reach three times the value that they had at the time of treatment— was calculated for each experimental assay group as follows. The relative volume of each tumor was calculated using the tumor volume at treatment as the standard. The proportion of the tumors which had reached three times the value of each standard was calculated for each group every day after the treatment. Data from these calculations were pooled for the analysis. A computer program based on the logit method of analysis was used. Standard statistical methods were used. Differences were considered significant if $p<0.05$. Animals with tumors that died of any cause before the tumor reached three times the standard value were not included in the analysis, and 7—13 mice were available for each group.

Table 1. shows the values of tumor growth time (X3). Tumor growth time (X3) for control was 3.3 days (single standard deviation was 0.6 days), for misonidazole 4.7 days (0.4 days), for hyperthermia 4.8 days (0.4 days), and for the combination of misonidazole with hyperthermia 5.5 days (0.6 days). The only significant difference in tumor growth time appeared between the control group and the misonidazole with hyperthermia group. From the results of the tumor growth times (X3), therefore, it was revealed that neither misonidazole (0.5 mg/g) nor hyperthermia (43°C, 10 min) had any tumor control effect but misonidazole with hyperthermia significantly delayed tumor growth.

4. TCD50 assays

Radiation dose response assays for tumor control at 90 days after irradiation were performed for 6 assay groups. The mice were randomly assigned to one of 6 radiation dose levels (average 9 mice per dose level) of one of the assay groups. X-ray irradiation and X-ray irradiation with misonidazole and/or hyperthermia was started when a tumor reached a mean diameter of 8 mm. The determinations were as follows; radiation only, radiation with hyperthermia (43°C, 10 min), radiation with misonidazole (0.5 mg/g mouse weight) and radiation with hyperthermia (43°C, 10 min) and misonidazole (0.5 mg/g mouse weight). A single dose of radiation was administrated at 30 minutes after the misonidazole injection, or without misonidazole (X-irradiation only or X-irradiation with hyperthermia). Hyperthermia was done simultaneously with X-irradiation. Animals were inspected twice a week for presence of tumor and tumor size. Animals with recurrent tumors bigger than 15 mm in diameter were sacrificed. Animals with tumors recurring at the

Table 1 Tumor growth times (X3)

	Tumor growth time (days)	s.d. (days)
Control	3.3	0.6
hyperthermia (43°C, 10 min)	4.8	0.4
misonidazole (0.5 mg/g body weight)	4.7	0.4
misonidazole + hyperthermia (0.5 mg/g) (43°C, 10 min)	5.5	0.6

Tumor growth time (X3)—days required for the tumors of 50% of the animals to reach three times the value that they had at the time of treatment.

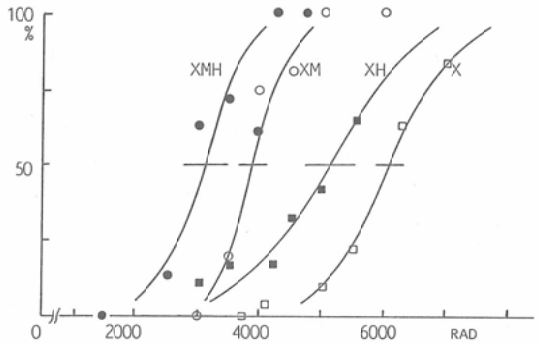

Fig. 4 Proportion of tumors controlled at 90 days vs X-ray dose. The vertical axis indicates the proportion of tumor controlled (%), and the transverse axis shows the X-ray dose. X—X-ray only; XH—X-ray + hyperthermia; XM—X-ray + misonidazale; XMH—X-ray + misonidazole and hyperthermia. The standard deviations of each curve are drawn in the figure at the level of 50%.

Table 2 TCD50 values and enhancement ratios

	TCD50 value (rad)	1S.D. (rad)	Enhancement ratio
X-ray only (control)	6024	286	1
X-ray + hyperthermia	5108	521	1.2
X-ray + misonidazole	3856	235	1.6
X-ray + misonidazole + hyperthermia	3093	319	1.9

margin of the irradiated field and those dying of any cause without apparent local recurrence were not included in the analysis. Data from several experiments were pooled for the TCD50 calculations. A computer program based on the logit method of analysis was used. The proportion of the controlled tumors at 90 days was calculated at each dose level of one of the assays. A total of 216 mice were available for the analysis.

The results of local control of FM3A tumors following irradiation in combination with misonidazole and/or hyperthermia are shown in Fig. 4 and Table 2. Fig. 4 shows the proportion of tumors controlled at 90 days vs X-ray dose. The sigmoid curves shown in the Fig. 4 were obtained by the logit method of analysis for each experimental group. Each curve shows the proportion of tumor control increase as the X-ray dose increases. The slopes of the curves relating the proportion of tumor control to X-ray dose are significantly different between control and misonidazole, and between control and misonidazole with hyperthermia.

The TCD50 value with control group (X-ray only) was 6024 rad (single standard deviation was 286 rad), 5108 rad (521 rad) with X-ray + hyperthermia (43°C, 10 min), 3856 rad (235 rad) with X-ray + misonidazole (0.5 mg/g mouse body weight), and 3093 rad (319 rad) with X-ray + misonidazole + hyperthermia. The combination of either misonidazole or hyperthermia with radiation enhanced tumor control compared with radiation used alone and the combination of the three modalities was the most effective.

The enhancement ratio (ER=the control group TCD50 value divided by the experimental group TCD50 value) for each experimental group is also shown in Table 2. A large value for the experimental group means that a lesser dose of X-ray is necessary to control the tumor to the same degree. The ER value was 1.2 for X-ray with hyperthermia, 1.6 for X-ray with misonidazole, and 1.9 for X-ray with misonidazole and hyperthermia, where it can be seen that the largest value was obtained with the combination of the three modalities.

Discussion

Hyperthermia (43°C, 10 min) and/or misonidazole (0.5 mg/g body wt) may have a slight effect on the tumor growth, but has no tumor control effect without X-irradiation in this study. The similar results with hyperthermia and/or misonidazole without irradiation on other in vivo tumor systems have been reported[4)5)14)].

The enhancement ratio for this tumor is 1.6 for misonidazole (0.5 mg/g mouse weight), which is smaller than that found for other tumors. In in vivo experimental tumors in C3H mice, local control was enhanced by misonidazole with an X-ray dose ratio of 1.82, where mice were treated with 1 mg/g body weight[23]. In an anaplastic MT tumor in a WHT/Ht mouse, the ER was 1.73 (0.3 mg/g. b.w.) and 2.08 (1.0 mg/g. b.w.)[13]. In a C3H mouse MDAH/MCa4 tumor, the ER was over 1.78 (0.3 mg/g. b.w.) and 2.30 (1.0 mg/g. b.w.)[11]. Few studies employed a dose of 0.5 mg/g. b.w. for misonidazole, therefore we cannot compare these values directly, but apparently the value for this tumor seems to be a smaller one.

Thrall et al.[6] found a ER of 1.39 when a C3H mammary carcinoma was treated in a 44.5°C waterbath for 15 minutes following irradiation in air. Stone[14] found a ER of 1.73 for heating at 42.5°C or 43°C for one hour which suggests that these doses of heat treatment are more effective than 44.5°C for 15 min. On the other hand, the ER values with hyperthermia have been measured for both skin and 7 types of transplantable tumors with intervals ranging from 0-24 hours and with heat given either before or after irradiation, where tumors were treated at 42.5°C for one hour in a waterbath[7][8]. Although the absolute thermal sensitization of tumors is greatest with hyperthermia treatments given directly after X-irradiation, a therapeutic advantage, considering normal tissue damage, is seen only with long intervals between X-irradiation and heating.

In this study, a simultaneous heating and irradiation treatment is employed, therefore simultaneous treatment was expected to produce a greater amount of skin damage compared with other procedures such as heating before or after irradiation which have been described in the previously mentioned reports. However, comparing the tumor control dose used in this experiment with those doses used in those treatments using only X-ray irradiation, there was no obvious difference in the skin reaction of the tissue around the tumor. It was thought that the side effects of hyperthermia on normal tissue would be small as heat was applied for a duration of only 10 minutes.

It this study, only the simultaneous effect of radiation and hyperthermia was studied, where the sequence and separation of the heat and X-irradiation were not in consideration. So no more discussion about a therapeutic gain value on the simultaneous effect of radiation and hyperthermia will be made.

Robinson et al.[24] reported that the therapeutic gain value is over 1.0 with tumor treated at below 43°C, but in contrast, Thrall et al.[6] reported that the therapeutic gain value is under 1.0 at 44.5°C. Overgaard and Suit[25] reported that the therapeutic gain factor was over 1.0 at under 43.5°C in considering "light" damage of normal tissue as acceptable, and 1.5 when "heavy" damage of normal tissue was acceptable. The ER value of 1.2 (43°C, 10 min) in this study seems to be comparable to these values.

The resultant ER value of 1.6 for misonidazole plus radiotherapy and 1.2 for heat pluse radiotherapy predicts an ER of 1.9 for combination therapy at 43°C for 10 min. if hyperthermia and misonidazole were acting independently in enhancing the radioresponse of the tumors. The observed value was 1.9. This suggests that at 43°C the two modalities may have been interacting with radiation independently. The ER at 43°C is consistent with a hypothesis of independence, but may also reflect enhanced cytotoxicity of misonidazole at the higher temperature. Hofer et al.[26] reported significant potentiation of cytotoxicity by combining the use of a hypoxic cell sensitizer with X-ray irradiation during hyperthermia.

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