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The Radiosensitizing Effects of Hypoxic Cell Sensitizer on SCCVII Tumors in Mice Evaluated by Highly Accurate Colony Formation Assay Assisted by Microcomputer

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マイクロコンピュータ支援による
高精度コロニーアッセイ法を用いた
SCCVII 腫瘍に対する
低酸素細胞増感剤の効果の検討

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電算機に支援された高精度コロニー形成率測定法で1-3Gyの放射線照射に対するSCCVII腫瘍の反応を調べた。細胞生存率は線量の増加とともに低下し、1-3Gy照射に対する固形腫瘍と培養細胞の放射線感受性を調べると後者のそれが有意に高かった。次に高線量照射時には同程度の増感効果を示すことが既に報告されている4種の低酸素細胞増感剤の効果を検討した。100mg/kg投与の場合、Etanidazole, KU-2285は腫瘍を増感しなかった。Misonidazoleは3Gyでのみ効果を示したが有意ではなかった($p=0.1$)。RP-170は2Gyでも3Gyでも効果を示し、3Gyの効果は有意であった($p<0.05$)。200mg/kg投与の場合、Etanidazole, Misonidazoleはともに放射線照射単独よりも低い生存率を示したが有意ではなかった。KU-2285とRP-170はともに有意な増感効果($0.01 < p < 0.05$, $p < 0.005$)を示したが、効果はRP-170の方が大きかった。低線量照射時の併用効果と高線量照射時の併用効果は異なることが明確に示された。

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Introduction

The radiation of larger than 4-5 Gy has been generally delivered to tumors in the research of chemical radiosensitizers, and many chemicals exhibited large enhancement ratios in mouse tumors. The clinical trials of hypoxic cell radiosensitizers were performed according to these data, however, the results have not been successful with some notable exception¹⁾ because of two probable reasons. The first is the intolerable toxicity of the drugs to humans at the dose level by which significant radiosensitization was achieved in mouse tumors. The second is the small radiosensitization achievable at the fraction dose of 2-3 Gy being prevalent in clinical radiotherapy. These findings highlight the importance of examining tumor responses following 1-3 Gy in experimental studies.

For assessing the tumor responses to low-dose radiation, multiple fractionated irradiation experiments have been employed using the 50% tumor control dose or tumor growth delay time as endpoints. In these experiments an immobilization of mice are required, and, this procedure may disturb the oxygenation status of the cells in tumors²⁾. On the other hand it is not necessary to restrict the mobility of mice when a standard *in vivo* - *in vitro* colony formation assay is applied for the study. However, the decreases of surviving cell fractions (SFs) following 1-3 Gy of radiation are too small to be accurately evaluated by standard *in vivo* - *in vitro* colony formation assay. Palcic et al³⁾ have established a sophisticated colony formation assay to examine the SFs and an exact oxygen enhancement ratios in dose ranges of less than 2 Gy in cultured cells.

As mentioned above, the radiosensitizing abilities of hypoxic cell sensitizers at low radiation doses have not been investigated in solid tumors of mice. Here, we applied the colony formation assay technique developed by Palcic for studying tumor responses to single dose of 1-3 Gy with or without four hypoxic cell radiosensitizers which have been reported to yield similar enhancement ratios when large radiation doses were delivered at single fraction.

Materials and Methods

Animals and tumors

SCCVII tumor cells in the exponential phase of growth in culture dishes were inoculated (5×10^4 cells) into the thighs of 8- to 10-week-old male C3H/He mice. The tumor cells were maintained in culture with Eagle's minimum essential medium supplemented with 12.5% fetal calf serum and 292 mg/l l-glutamine. About 2 weeks later, when the studies were performed, the tumors reached a mean diameter of 10 mm.

Irradiation

The radiation was delivered to the whole body of tumor-bearing mice at dose rates of 0.95-1.0 Gy/min, using an irradiator loaded with 370 TBq ^{60}Co . Dose rate meter was used to measure the absorbed dose rate. Four mice were irradiated at once in an acrylic box (volume = 784 ml). The mice were not immobilized or subjected to general anesthesia. To determine the *in vitro* radiosensitivity of tumor cells, 15 ml centrifuge tubes containing tumor cell suspension were placed in a same box for irradiation to mice, and radiation was delivered.

Compounds

Four hypoxic cell radiosensitizers: Etanidazole, Misonidazole, 1-(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl-2-nitroimidazole (RP-170)⁴⁾ and 2-nitroimidazole with an N¹-substituent of $\text{CH}_2\text{CF}_2\text{CONHCH}_2\text{CH}_2\text{OH}$: KU-2285⁵⁾ were tested for their radiosensitizing activity. The drugs were dissolved in physiological saline, and 100 or 200 mg/kg body weight was administered intravenously. Twenty minutes later the mice received whole body irradiation of 1-3 Gy. These drug doses and an interval between drug injection and irradiation were chosen according to the previous studies by others^{4),5)}.

Colony Formation Assay

Immediately after the treatments, the mice were killed, and the tumors were excised and trypsinized (0.05% trypsin + 0.02% EDTA at 37°C for 15 min). The tumor cell suspension was divided into two samples. One was for examining the adhesion rates of tumor cells to the flask bottom. The other was provided for colony formation assay. Four to five thousands tumor cells were plated onto T-25 flask for determining the ratios between the number of cells adhered to the bottom of flask and the number of cells inoculated. Cytochalasin B, a cytokinesis inhibitor, was added to the medium (2 µg/ml). Eight to ten hours later, all tumor cells which existed in a pre-marked area of the bottom were counted and the culture medium was exchanged. Immediately after, the cells still remaining in the same area of the

bottom were counted again to determine the adhesion rates of tumor cells.

For the colony formation assay, around 400 tumor cells obtained from the remaining sample of tumor cell suspension were plated onto a T-25 flask. Parallel lines, at 2-mm interval, were pre-marked on the outer surface of bottom of flask. Eight to ten hours later, the medium was exchanged, and the flasks were set on a specially designed table of the phase contrast reversed microscope (Fig. 1 A), and the bottoms were carefully observed scanning along the parallel lines. About 100 positions where tumor cells adhered to the bottom were recorded in a microcomputer. Fig. 1 B shows the operation console involving the microcomputer for recording cell positions, and the positions can be revisited automatically every time when it is needed. The parallel lines completely eliminated the risk of dual recording of cell positions. After 5 to 6 day culture, the colonies in the flasks were fixed with Carnoy's fixative:

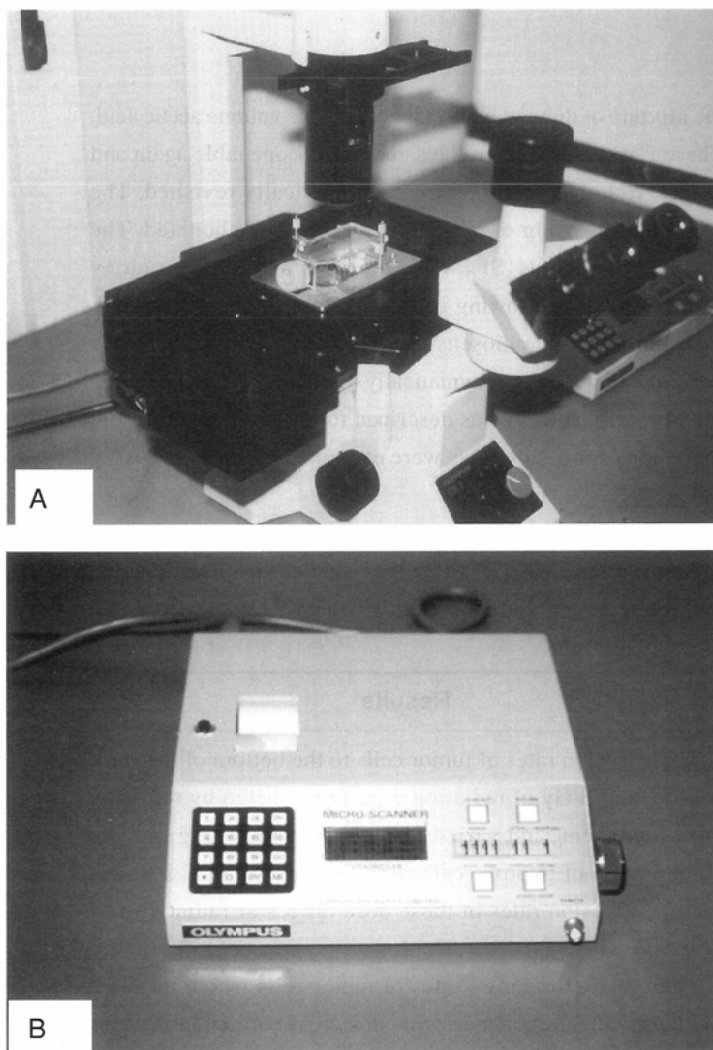


Fig. 1 Phase contrast reversed microscope (A) with a specially designed table connected to operation console (B) which is involving microcomputer for recording cell positions. Every prerecorded positions can be revisited automatically every time when it is needed.

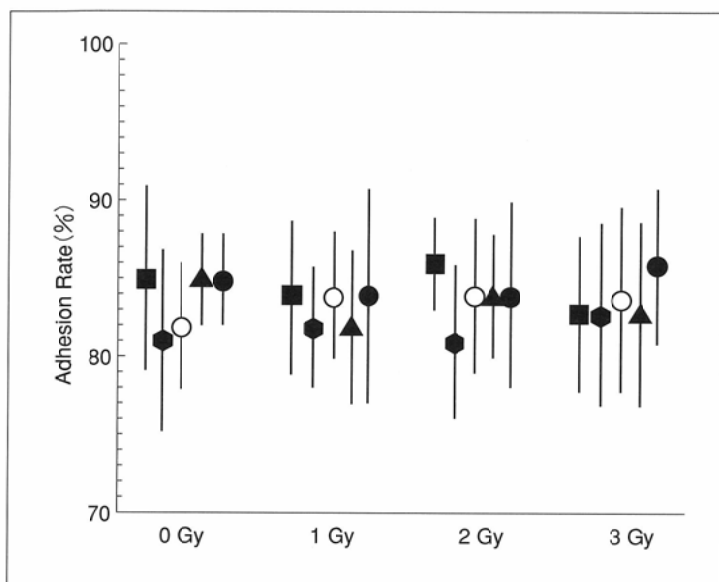


Fig. 2 Rate of adhesion of tumor cells as a function of radiation dose. Results represent mean values and standard deviations in seven independent experiments. (○) : Radiation alone, (●) : Misonidazole, (▲) : Etanidazole, (■) : KU-2285, (◆) : RP-170.

the mixture of three volume ethanol and one volume acetic acid. These flasks were put on the same microscope table again and all pre-recorded positions were automatically revisited. The colonies consisting of more than 50 cells were counted. The surviving fractions (SFs) were the ratio divided the colony formation rates following irradiation by that of unirradiated. To determine the radiosensitivity of fully oxygenated tumor cells, the tumor cells enzymatically dissociated from untreated tumors were irradiated as described in former paragraph, and the colony formation rates were examined by the same assay procedure.

The difference between SFs was statistically analyzed by Student's t-test. When P value was smaller than 0.05, the difference was regarded as statistically significant.

Results

The adhesion rates of tumor cells to the bottom of the flask following 0-3 Gy of radiation were not affected by radiation dose increment, and varied from 77% to 91% (Fig. 2). The combination of hypoxic cell radiosensitizers had also no effect on adhesion rates in these dose ranges of radiation and drugs (Fig. 2).

The colony formation rates of tumor cells which received radiation *in vivo* decreased with an increase of radiation doses in the range of 0-3 Gy (Fig. 3). The tumor cells irradiated *in vitro* exhibited higher radiosensitivity than those treated *in vivo*. The radiosensitizing effects of the hypoxic cell radiosensitizers on tumors at the dose of 100 mg/kg are pre-

sented in Fig. 4. Etanidazole and KU-2285 did not enhance the effect of radiation to tumors. The combination of misonidazole and radiation reduced the SF slightly more than that with radiation alone at only 3 Gy, however, this difference was not significant ($p = 0.1$). Irradiation combined with RP-170 yielded a lower SF at 2 Gy than that with radiation alone ($0.05 < p < 0.1$). At 3 Gy, RP-170 significantly increased the tumor cell killing of radiation ($p < 0.05$).

Fig. 5 demonstrates the combined effects of radiation and radiosensitizers at the dose of 200 mg/kg. The mean SFs in the combination of radiosensitizers and radiation were lower than those achieved with radiation alone. However, the decreases of SFs were not significant in the cases of misonidazole and etanidazole. The combination of KU-2285 and radiation yielded lower SFs at 2 and 3 Gy in comparison with radiation alone, and these differences were significant ($0.01 < p < 0.05$). The SFs following 2 and 3 Gy of radiation combined with RP-170 were significantly lower than each that produced by radiation alone ($p < 0.005$).

Discussion

Accuracy of microcomputer assisted colony assay for determining *in vivo* tumor response to 0-3 Gy

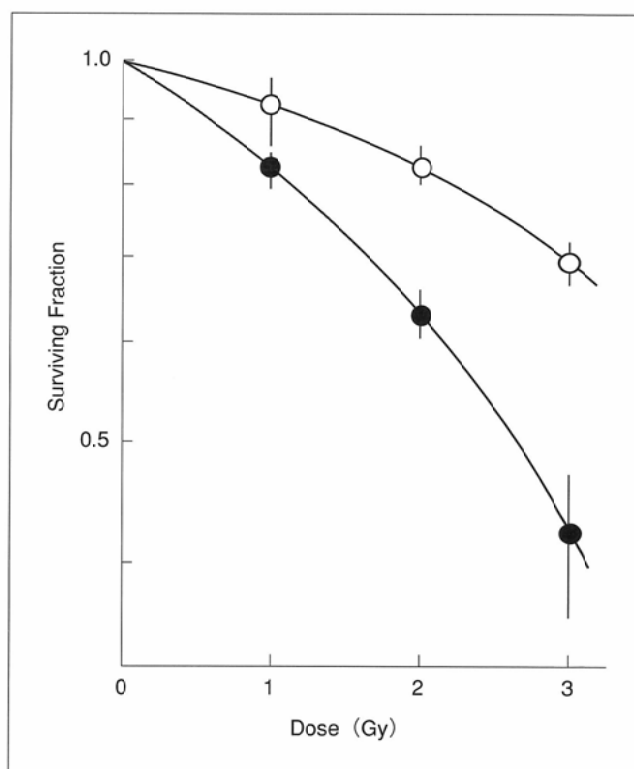


Fig. 3 Surviving cell fraction as a function of radiation dose. (○) : *in vivo* radiation, (●) : *in vitro* irradiation. Each point indicates mean value and standard deviation in seven and four independent experiments of *in vivo* and *in vitro* irradiation, respectively.

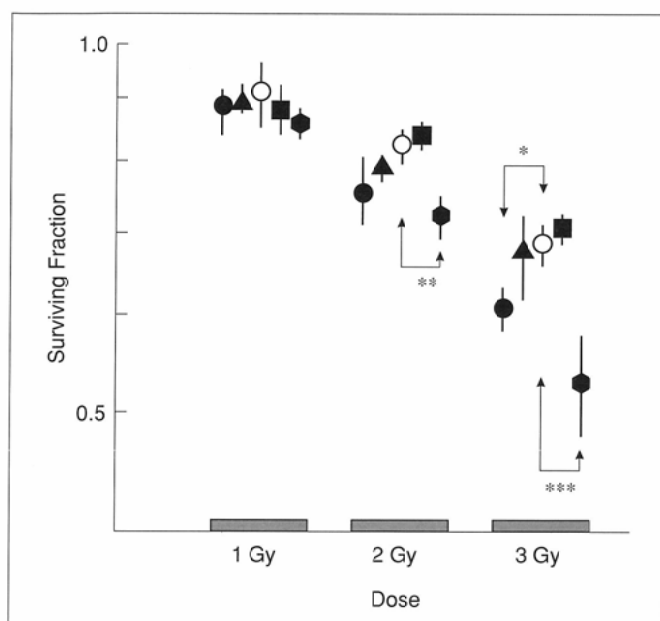


Fig. 4 Surviving cell fractions of tumors irradiated *in vivo* as a function of radiation dose with 0.1 mg/kg of hypoxic cell radiosensitizers. (○): radiation alone, (●): Misonidazole, (▲): Etanidazole, (■): KU-2285, (●): RP-170. Each point indicates mean value and standard deviation of 10 flasks in five independent experiments. *: $p = 0.1$, **: $0.05 < p < 0.1$, ***: $0.01 < p < 0.05$

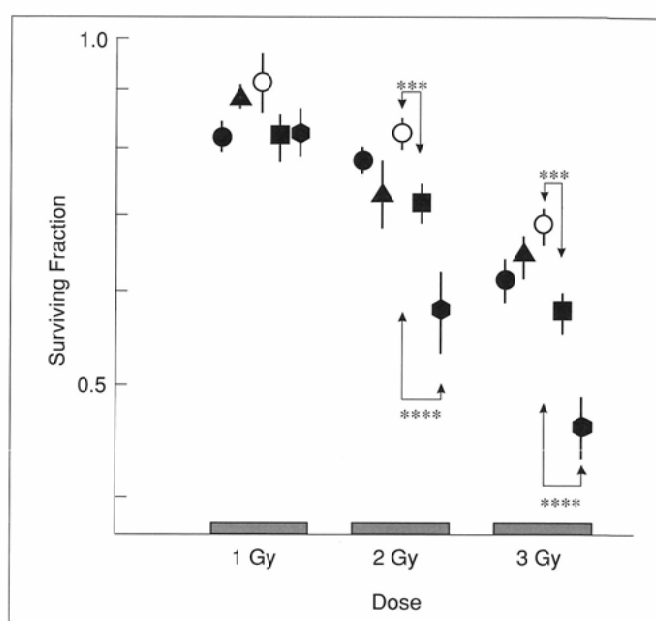


Fig. 5 Surviving fractions of tumors irradiated *in vivo* as a function of radiation dose with 0.2 mg/kg of hypoxic cell radiosensitizers. (○): radiation alone, (●): Misonidazole, (▲): Etanidazole, (■): KU-2285, (●): RP-170. Each point indicates mean value and standard deviation of 10 flask in five independent experiments. ***: $0.01 < p < 0.05$, ****: $p < 0.005$.

The cell position prerecorded colony formation assay assisted by microcomputer was developed by Palcic et al³⁾ to investigate the oxygen enhancement ratio for cultured cells at 2 Gy region. This colony formation assay procedure has also been used to examine the repair phenomenon which are inducible by very low dose radiation (<1.0 Gy)⁶⁾. As described above, this assay technique has been approved as highly accurate method for an assessment of *in vitro* radiosensitivity of cells. On the other hand, in conventional *in vivo-in vitro* colony formation assay, it is difficult to detect tumor response to low dose of radiation, because this assay involves the errors in cell counting, dilution of cell suspension, cell plating onto flask and inter-tumor variations. When this new technique would be applied to *in vivo-in vitro* colony assay, however, the errors described above could be essentially eliminated except the inter-tumor variations.

Adhesion rate of tumor cells following radiation

In the *in vitro* study, the cells were plated, and their adhesion positions were recorded. Thereafter, radiation was delivered to the cells. However, for assessing the responses of *in vivo* tumors, radiation must be delivered first. If the adhesion of tumor cells to the bottom of flask is affected by radiation, the technique used here can't be simply applied to evaluate the response of *in vivo* tumors. We therefore examined the effects of radiation on adhesion rates of tumor cells. As presented in Fig. 2, these values did not decreased with an increase of radiation doses, probably due to the low doses of radiation in

our study.

Surviving cell fractions of tumors following 0-3 Gy

The colony formation assay revealed the slight tumor responses following 1-3 Gy, clinically prevalent as fraction dose (Fig. 3). Tumor consists of well oxygenated radiosensitive and hypoxic radioresistant cells. The hypoxic cell fraction of SCCVII tumors at a mean diameter of 10 mm is around 14%⁷⁾. The radiation response of tumors at low doses is mainly determined by the killing of well oxygenated tumor cells⁸⁾. Therefore, it is generally thought that the cell survivals of tumors at low doses does not differ from those of tumor cells irradiated *in vitro*. However, in the present study, there were significant differences in tumor cell survivals between tumors irradiated *in vivo* and tumor cells received *in vitro* irradiation (Fig. 3). In our previous study using micronucleus assay, we found that the combination of nicotinamide and carbogen inhalation enhanced the response of SCCVII tumors to 1-4 Gy being equivalent to the radiation doses in the present study. Because nicotinamide decreases the cyclic interruption of microregional tumor blood flow⁹⁾ and carbogen inhalation increases the oxygen partial pressure in tumor tissue¹⁰⁾, the increased tumor responses are regarded as an improvement of oxygenation status of the tumors. Therefore, our present finding is consistent with previous report¹¹⁾.

Effects of hypoxic cell radiosensitizers

Some investigators have studied the effects of hypoxic cell radiosensitizers in combination with multiple fractionation

irradiation by conventional *in vivo* - *in vitro* colony formation assay. However, the fraction doses applied were not around 2 Gy, but above 4 Gy¹²⁾. Of course multiple irradiation experiments using 2 Gy fraction is possible, however, the final effects on tumors are affected by many factors: the initial size of damage, the extent of recovery between irradiations, reoxygenation, repopulation and so on. To exclude the effect of reoxygenation, the clumping of tumors prior to each radiation exposure was employed and the radiosensitizing activity was determined on pure hypoxic cells¹³⁾. However, the effect which have to be known is that on a mixed tumor cell population consisting of both oxic and hypoxic cells. Then, we used microcomputer assisted assay technique to test the activity of hypoxic cell radiosensitizers and found that, of the 4 agents tested, only RP-170 was effective at 100 mg/kg in combination with 3 Gy of radiation (Fig.4). The combination of RP-170 with 2 Gy yielded a slightly lower SF than that with radiation alone, however, the difference was not significant ($0.05 < p < 0.1$). RP-170 may have potential for increasing tumor response at clinically prevalent fraction dose of 2-3 Gy. When

200 mg/kg of KU-2285 or RP-170 was combined with 2-3 Gy, significant enhancement of cell killing was obtained (Fig.5). According to the studies performed by others, these four radiosensitizers have significant radiosensitizing activity at the drug doses of 100 or 200 mg/kg when large radiation doses were combined^{4),5),14)}. However misonidazole and etanidazole did not show such an activity at 2 - 3 Gy, and RP-170 had a more significant effect than KU-2285, despite their similar activities at large radiation doses^{4),5)}. Thus, the radiosensitizing activity at around 2 Gy did not always correlate with that observed at large radiation doses.

Conclusion

KU-2285 and RP-170, especially RP-170 increased the radiation response of tumors which are consisting of heterogeneous mixed cell populations concerning oxygenation status at the drug dose of 100 mg/kg and 2 Gy of radiation. Therefore, we consider that RP-170 and KU-2285 have potential to increase tumor response in clinical radiotherapy.

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