Time Course of Reoxygenation in Experimental Murine Tumors after Carbon-beam and X-ray Irradiation

NATSUO OYA1*, KEISUKE SASAI1, TORU SHIBATA1, TAKEHISA TAKAGI1, KEIKO SHIBUYA1, SACHIKO KOIKE2, KUMIE NOJIMA2, YOSHIYA FURUSAWA2, KOICHI ANDO2 and MASAHIRO HIRAOKA1

1Department of Therapeutic Radiology and Oncology, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606–8507, Japan
2International Space and Radiation Laboratory, National Institute of Radiological Sciences, 9–1 Anagawa-4-chome, Inage-ku, Chiba-shi, Chiba 263–8555, Japan

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We compared the tumor reoxygenation patterns in three different murine tumor cell lines after X-irradiation with those after carbon-beam irradiation using a heavy-ion medical accelerator (HIMAC) system. The tumors of the cell lines SCCVII, SCCVII-variant-1 and EMT6 on the hind legs of mice received local priming irradiation with a carbon-beam (8 Gy, 73 keV/µm in LET, 290 MeV/u, 6 cm SOBP) or X-rays (13 Gy, 250 kVp). After various intervals, the mice were given whole-body test irradiation (16 Gy, 250 kVp X-ray) either in air or after they were killed. The hypoxic fractions were estimated as the proportions of the surviving fractions of the tumors in killed mice to those in air-breathing mice. In the SCCVII tumors, the hypoxic fractions at 0.5 h were 50% and 21% (p < 0.05) after the priming X-irradiation and carbon-beam irradiation, respectively. In the SCCVII-variant-1 tumors, the hypoxic fractions were 85% and 82% at 0.5 h, 84% and 20% at 12 h (p < 0.01), and 21% and 31% at 24 h after X-ray and after carbon-beam irradiation, respectively. In the EMT6 tumors, the reoxygenation patterns after X-irradiation and carbon-beam irradiation were quite similar. We concluded that the reoxygenation pattern differed among the three tumor cell lines, and that reoxygenation tended to occur more rapidly after carbon-beam irradiation than after X-irradiation for SCCVII and SCCVII-variant-1 tumors.

INTRODUCTION

Hypoxic cells are a potential cause of the failure of a radiotherapeutic treatment for...
tumors. Several previous studies using animal tumor models have demonstrated the existence of clonogenic hypoxic cells, and their influence on the radiosensitivity of a tumor. Various methods for measuring hypoxia, such as oxygen microelectrodes, nitroimidazole binding assays, and paramagnetic resonance oximetry, have been established, and findings obtained with these techniques suggest that human tumors also contain hypoxic cells, although the degree of the impact of hypoxia on the human tumor response is still being investigated.

In clinically relevant fractionated photon radiotherapy, the reoxygenation procedure which may occur between two fractions could increase the probability of tumor control. However, if the surviving hypoxic cells have reoxygenated only insufficiently at the time of the second fraction, the benefit of a fractionated regimen might be decreased. Therefore, the time course of reoxygenation is one of the most important factors which influence the probability of tumor control after fractionated radiotherapy.

Heavy-ion particle radiation therapy is one of the most promising modalities to treat human tumors, since its radiobiological characteristics, as well as its precise dose-localization characteristics, may offer an advantage in the radiotherapy of tumors. Clinical investigations of heavy-ion particle radiation therapy for human tumors using a heavy-ion medical accelerator (HIMAC) system have been underway at the National Institute of Radiological Sciences (NIRS), Chiba, Japan since 1993. Simultaneously, many experimental studies are also being undertaken in several laboratories to investigate the radiobiological features of heavy-ion beams generated by HIMAC concerning, for example, the relative biological effects, apoptosis, repopulation, and potential lethal damage repair. How reoxygenation occurs after heavy-ion particle irradiation is also of great concern, because heavy-ion particle irradiation may be used in clinical practice as a combined modality with photon irradiation, chemotherapy, or hyperthermia, in which the tumor oxygenation status is one of the critical problems. It is well known that high linear energy transfer (LET) radiation has a low oxygen enhancement ratio (OER). However, the degree of hypoxic cell reduction in the in-vivo tumors induced by heavy-ion particle radiation and the following reoxygenation pattern has not yet been fully examined.

In the present study, we compared the tumor reoxygenation patterns in three murine tumor cell lines after X-irradiation with those after carbon-beam irradiation by the HIMAC.

**MATERIALS AND METHODS**

**Tumor cell lines and animals**

Three tumor cell lines (SCCVII, SCCVII-variant-1, and EMT6) were used in the present experiments.

SCCVII squamous cell carcinoma and EMT6 mammary sarcoma were maintained alternately in-vitro and in-vivo, and used in many series of experiments in our laboratory as established and stable cell lines. Their characteristics are described elsewhere. EMT6 cells and SCCVII cells have comparable in-vitro radiosensitivity, and EMT6 has slightly shorter
in-vitro and slightly longer in-vivo doubling times compared with that of SCCVII. The third cell line used, SCCVII-variant-1, was separated from SCCVII in our laboratory in 1988 by repeated in-vitro subcultures of SCCVII. The SCCVII-variant-1 cells had been stored frozen in liquid nitrogen, and were used in the present experiments after an interval of 8 years. As preliminary experiments, the tumor growth and radiosensitivity of SCCVII and SCCVII-variant-1 were compared using their in-vitro cultures and in-vivo tumors in C3H/He mice. SCCVII-variant-1 has some different characteristics from the original SCCVII (see Results section).

The cells from each cell line were cultured in Eagle’s minimum essential medium containing 12.5% fetal bovine serum (FBS) throughout the experiments. SCCVII and SCCVII-variant-1 cells were inoculated subcutaneously into the right hind legs of syngeneic 7–9-week-old female C3H/He mice. EMT6 cells were inoculated subcutaneously into the right hind legs of syngeneic 8 week-old female Balb/c mice. In-vivo experiments using SCCVII, SCCVII-variant-1, and EMT6 were carried out when the tumors reached 6 mm, 6 mm, and 7 mm in average diameter, 7–9 days, 7–9 days, and 8–10 days after inoculation of $2 \times 10^5$, $6 \times 10^5$, and $4\text{--}5 \times 10^5$ exponentially growing cells from in-vitro monolayer cultures, respectively. In the present study, we used these rather small tumors to make the LET and dose distribution within each tumor equal. Gross necrosis was rarely seen.

**Irradiation**

Carbon-beams were generated by HIMAC, constructed at NIRS\textsuperscript{16). Throughout the experiments using carbon-beam irradiation, 290 MeV/u beams of 73 keV/µm in LET with a 6-cm width spread-out bragg peak (SOBP) were used. The dose rate ranged from 2–4 Gy/min.

X-rays were produced by a 250 kVp X-ray unit (PANTAK MI-201) at 16 mA with a filtration of 0.5 mm Cu at a dose rate of 0.9–1.5 Gy/min.

The tumors received a local priming 13-Gy dose of X-rays or 8-Gy dose of carbon-beams. The tumor-bearing legs of unanesthetized mice were fixed in individually designed acrylic jigs with adhesive tape to enable the tumor to receive local dorsoventral irradiation\textsuperscript{22,23).}

After various intervals, the mice were given a whole-body test 16-Gy dose of X-rays in an acrylic box, either alive in an air-breathing condition without anesthesia, or 5 min after being killed by cervical dislocation\textsuperscript{22}. For the test irradiation, whole-body irradiation was performed to avoid an artificial increase in the hypoxic fraction due to fixation\textsuperscript{24). The intervals from the priming irradiation to the test irradiation were 0.5, 1, 6, 12, 24 and 72 h, or with slight modifications. Because the experiments were repeated four times, at least four mice were used for one experimental point in most cases.

**Determination of hypoxic fraction**

The surviving fractions were determined using our standard in vivo-in vitro colony assay, as described in detail previously\textsuperscript{3,4,6,22,23). Briefly, immediately after the test irradiation, the tumors were excised, minced, and magnetically stirred with 0.1% neutral protease for 40 min
at 37°C to obtain single-cell suspensions. Known appropriate numbers of cells were plated, and 10–11 days later were fixed and stained with Giemsa to count colonies.

The hypoxic fractions at each time point were estimated as the proportions of the surviving fractions of the tumors in killed mice to those in air-breathing mice, instead of using the conventional paired survival-curve method\(^{1–4,6,7,9,23}\). We assumed that the hypoxic fractions determined by this simplified method should approximate those determined by the conventional method, since previous reports showed that a test dose of 16 Gy is within the dose range of the hypoxic tail; i.e., large enough to sterilize nearly all of the oxic cells in the tumors\(^{1,4,6}\). To determine the hypoxic fractions in the untreated tumors, only a test dose of 16 Gy was irradiated. A Student’s t-test was used to examine the difference between the pairs of hypoxic fractions.

Fig. 1. Comparison between SCCVII and SCCVII-variant-1. (a) In-vitro growth curves. (b) Tumor growth curves following the subcutaneous inoculation of 4 × 10\(^5\) cells. The tumor volume was determined while assuming an ellipsoid shape of \(\pi x y (z-u)/6\), where \(x, y, z,\) and \(u\) represent the cephalocaudal diameter, the lateral diameter of the tumor, the thickness of the tumor-bearing leg, and the non-tumor-bearing leg, respectively. (c) In-vitro surviving curves. (d) In-vivo surviving curves for 6 mm tumors irradiated in air-breathing mice. The error bars are 1 S.E. from 3 to 4 independent experiments for (a), (c), and (d), and from 9 mice for (b).
RESULTS

Figure 1 shows the results of preliminary experiments performed to compare the characteristics of SCCVII and SCCVII-variant-1. In the in-vitro culture, both of them grew exponentially, with the latter growing slightly more rapidly than the former. However, their in-vivo tumor growth curves following subcutaneous inoculation of $4 \times 10^5$ cells were quite different. The SCCVII-variant-1 tumors increased in size rapidly until Day 10, thereafter began to decrease in size, and disappeared by Day 19. None of the observed 9 tumors continued to grow. The SCCVII tumors grew exponentially at least up to Day 18. The SCCVII tumor cells were more radiosensitive than and as radiosensitive as SCCVII-variant-1 when irradiated under the in-vitro and in-vivo conditions, respectively.

The mean plating efficiency was 17% for the SCCVII tumors, 28% for the SCCVII-variant-1 tumors, and 54% for the EMT6 tumors.

Figures 2, 3, and 4 show the surviving fractions and the hypoxic fractions in the SCCVII, SCCVII-variant-1, and EMT6 tumors, respectively, as a function of the interval from the priming irradiation to the test irradiation.

SCCVII (Fig. 2): The hypoxic fraction in untreated 6-mm SCCVII tumor was 14%. Fol-

![Fig. 2. Reoxygenation in 6-mm SCCVII tumors. (a) Surviving fraction after a priming 13-Gy dose of X-rays and a test 16-Gy dose of X-rays. (b) Surviving fraction after a priming 8-Gy dose of carbon-beams and a test 16-Gy dose of X-rays. (c) Hypoxic fraction as a function of the treatment interval between the two doses. □ Mice were air-breathing during test irradiation. □ Mice were killed 5 min before test irradiation. □ X-ray (13 Gy). □ Carbon-beam (8 Gy). The broken line represents the hypoxic fraction in untreated tumors. The error bars are 1 S.E. from 3 to 6 independent experiments. * p < 0.05.](https://academic.oup.com/jrr/article-abstract/42/2/131/959446)
Fig. 3. Reoxygenation in 6-mm SCCVII-variant-1 tumors. See the legend for Fig. 2. The error bars are 1 S.E. from 4 independent experiments. ** p < 0.01.

Fig. 4. Reoxygenation in 7-mm EMT6 tumors. See the legend for Fig. 2. The error bars are 1 S.E. from 2 to 5 independent experiments.
lowing the priming X-irradiation and carbon-beam irradiation, the hypoxic fraction at 0.5 h was 50% and 21%, respectively (p < 0.05). In both cases, the hypoxic fraction gradually decreased, approaching the control level by 12 h.

SCCVII-variant-1 (Fig. 3): The hypoxic fraction in the untreated 6-mm SCCVII-variant-1 tumors was 19%. The hypoxic fraction value at 0.5 h after the priming carbon-beam irradiation (82%) was as high as that after the priming X-irradiation (85%). However, the reoxygenation patterns were quite different. The hypoxic fraction remained at about 85% within the first 12 hours after the priming X-irradiation, and fell to the control level at 24 h. In contrast, after the priming carbon-beam irradiation, the hypoxic fraction began to decrease within the first 1 h, substantially decreased until 12 h, reached the control level by 24 h, and fell to below the control level at 75 h. Consequently, these two curves were vertically distinct from each other from 1 h to 12 h. The hypoxic fraction value at 12 h after the X-irradiation was significantly higher than that after the priming carbon-beam irradiation (84% vs. 20%, p < 0.01).

EMT6 (Fig. 4): The hypoxic fraction in the untreated 7-mm EMT6 tumors was 19%. The reoxygenation patterns after X-irradiation and carbon-beam irradiation were quite similar. In both cases, the hypoxic fraction substantially decreased in fluctuation until 24 h, and did not return to the control level at least up to 77 h, which was the maximal interval examined. The hypoxic fraction values after X-irradiation and carbon-beam irradiation were not significantly different at any time point.

**DISCUSSION**

The magnitude and time course of reoxygenation after photon irradiation have been investigated in many experimental tumor models, revealing that the reoxygenation pattern following irradiation as well as the hypoxic fraction in untreated tumors could vary with the tumor type, tumor size, tumor conditions at irradiation, and dose or dose fractionation. The present data suggested that the reoxygenation pattern also differed among the examined three tumor cell lines. The difference was most obviously shown between SCCVII and SCCVII-variant-1, which reoxygenated rapidly and slowly after X-irradiation, respectively, in spite of their comparable in-vivo sensitivities to X-rays.

Whether the reoxygenation pattern depends on LET has not been fully investigated. In the present study, more rapid reoxygenation after a 8-Gy dose of carbon-beams than after a 13-Gy dose of X-rays was observed in two of the three cell lines, SCCVII and SCCVII-variant-1. Although 8 Gy of carbon-beams and 13 Gy of X-rays may not be exactly identical in the cytokingilling effect, it seemed to be appropriate to consider that the differences in the time course of reoxygenation were mainly due to the difference in the LET.

SCCVII tumors reoxygenate by nature very rapidly. When the two reoxygenating curves of the SCCVII were compared, the only significant difference between the carbon-beam and X-ray irradiation was observed at 0.5 h after the primary irradiation. Considering that the hypoxic fraction at 0.5 h after the primary X-irradiation was only 50%, and that at 0.5 h
after the primary carbon-beam irradiation was almost at the control level, higher values of the hypoxic fraction or a greater difference between carbon-beam and X-ray irradiation might be expected before 0.5 h. However, under the present experimental condition, the measurement of the hypoxic fraction after a shorter interval was technically difficult.

The influence of LET on the rate of reoxygenation was demonstrated most conspicuously in SCCVII-variant-1 tumors. As mentioned above, this unintentionally established cell line seemed to have acquired several characteristics quite different from those of the original SCCVII. The shape of the reoxygenation curve following the priming X-irradiation, as well as the hypoxic fraction value for the untreated tumors, of the SCCVII-variant-1 were similar to those of the EMT6 rather than the original SCCVII. Even after the carbon-beam irradiation, the SCCVII-variant-1 tumors reoxygenated slowly, returning to the preirradiation level at 12 h, but not as slowly as after the X-irradiation. The hypoxic fraction at 0.5 h after the primary carbon-beam irradiation was high (82%), suggesting that the OER of the carbon-beam irradiation for SCCVII-variant-1 was not much smaller than that of X-rays.

In EMT6 tumors, there seemed to be no difference between the reoxygenation patterns after the carbon-beam irradiation and the X-irradiation. It is unlikely that a greater difference is missed before 0.5 h, because the hypoxic fraction at 0.5 h after the carbon-beam irradiation was considerably high (79%).

For at least the two tumor cell lines examined in the present study, SCCVII and SCCVII-variant-1, it is suggested that reoxygenation occurs after carbon-beam irradiation more rapidly than after X-irradiation. One of the arguments used to explain the difference observed is the reduced dependence on the oxygenation status for cell killing (i.e., low OER) in high-LET irradiation2,18,19,26). According to previous studies using a rat rhabdomyosarcoma tumor, the procedure of reoxygenation might not play an important role after carbon-beam (BEVALAC, 400 MeV/u in initial energy) irradiation, because of the minor initial increase in the hypoxic fraction due to its OER, as low as 1.918,19). A recent study using V79 and human salivary gland tumor cells has shown that the OER values of the carbon-beams with LET of around 73 keV/µm were as low as 2–2.527). Although the OERs for the cell lines used in the current experiments were not precisely examined, it should have been at least to some extent lower than that of 250 kVp X-rays. For the SCCVII tumors, in which the hypoxic fraction at 0.5 h after the carbon-beam irradiation was 21%, the “minor initial increase in the hypoxic fraction” could account for the apparently rapid reoxygenation after the priming carbon-beam irradiation. However, considering the reoxygenation curve for the SCCVII-variant-1 and EMT-6 tumors, in which the hypoxic fraction at 0.5 h after the carbon-beam irradiation was as high as 80%, this argument does not always fully explain the difference between the time course of reoxygenation after the carbon-beam irradiation and that after X-irradiation.

Another possible explanation regards the behavior of damaged cells, which are fated to die, since rapid cell death, including apoptosis, can be one of the major causes for rapid reoxygenation. Following photon irradiation, the cells which received low-LET-type lethal damage continue to live and consume oxygen, at least until subsequent mitosis. They then fail to divide and are usually destroyed during mitosis, though it is also possible that those cells with fatal, but relatively slight, damage may divide to produce daughter cells28). These cells
continue to consume oxygen, duplicating DNA and synthesizing proteins for several cell
cycles until being destroyed.

On the other hand, the damage brought about by high-LET radiation is usually so dense
and complicated that the damaged cells can neither continue metabolism nor pass through the
cell cycle. In this situation, only a small amount of oxygen seems to be consumed for only a
short period by the cells which are fated to die. Accordingly, since oxygen diffusion toward
the surviving hypoxic cells occurs more efficiently, reoxygenation starts even before the blood
perfusion starts to increase. This hypothetical mechanism of rapid reoxygenation after high-
LET irradiation seemed to be similar to the previously reported mechanism of rapid
reoxygenation of SCCVII tumors after photon irradiation.

Several studies have been performed to assess the relative biological effectiveness (RBE)
of high-LET radiation with special respect to apoptosis, mostly using apoptosis-liable cells.
Still, it remains controversial as to whether apoptosis is dependent on the LET. The RBE for
apoptosis was reported to be 1.0 in mouse thymocytes and in human peripheral lympho-
cytes irradiated with neutrons, while it was reported to be 1.3–4 in intestinal crypt cells
irradiated with neutrons, in murine T lymphoma cells irradiated with alpha particles and
human peripheral lymphocytes irradiated with nitrogen beams. Further, a recent study using
V79 cells suggested that the RBE of the carbon-beams with an LET of 73 keV/µm was
between 1.4 and 1.8. Although these data can not be simply applied to the murine tumor
cell lines used in the present study, if apoptosis occurs commonly following high-LET-type
DNA damage, it should allow the damaged cells to die rapidly, and should contribute to rapid
reoxygenation. However, since the incidence of radiation-induced apoptosis in these tumors is
usually very low, the LET dependency of the reoxygenation pattern can be only partly
explained by apoptosis. It is probable that there are some in-vivo specific factors which may
affect reoxygenation. Therefore, the in-vivo behavior of cells after carbon-beam irradiation, as
well as apoptosis or the OER for carbon-beam irradiation, should be further examined.

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