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Monitoring of response to radiation therapy for human tumor xenografts using ^{99m}Tc-HL91 (4,9-diaza-3,3,10,10-tetramethyldodecan-2,11-dione dioxime)

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Purpose: Oxygenation status of tumor tissue is an important factor to discriminate it with respect to its radiosensitivity. ^{99m}Tc-4,9-diaza-3,3,10,10-tetramethyldodecan-2,11-dione dioxime (^{99m}Tc-HL91) is retained in hypoxic tissues, making it possible to use it as hypoxic imaging agent. We evaluated if the accumulation of ^{99m}Tc-HL91 in tumors could aid in the prediction of sensitivity of radiation therapy of cancers. ***Methods:*** Human tumors (the gastric cancer cell line: MKN45, the epidermoid carcinoma cell line: KB-31, and the lung adenocarcinoma cell line: HLC) were xenografted into the thigh of athymic mice and irradiated with a 4 MV linear accelerator. Tumor growth was measured and ^{99m}Tc-HL91 uptakes in tumors were determined by serial imaging, biodistribution, and autoradiography. ***Results:*** ^{99m}Tc-HL91 uptake (ratio of ROI_{tumor} to $ROI_{whole\ body}$) in HLC ranged from 1.1 to 8.0%, and it did not show any response to radiation therapy. Major variations were observed in ^{99m}Tc-HL91 accumulation in MKN45 and KB-31; from 0.7 to 4.7%, and from 1.0 to 7.3%, respectively. Some tumors responded to radiotherapy, while others did not. Tumor response was not dependent on the ^{99m}Tc-HL91 uptake, tumor size or radiation dose. Comparing ^{99m}Tc-HL91 uptake in tumors before (B) and after (A) their radiation, uptake (B) was always smaller than uptake (A) for HLC, and they did not respond to irradiation at all. For MKN45 and KB-31, tumors responded to radiation when their uptake (A) was not higher than uptake (B). In contrast, the tumors continued to grow when their uptake (A) was higher than uptake (B). Sequential ^{99m}Tc-HL91 imaging of KB-31 and their autoradiography indicated that tumors whose ^{99m}Tc-HL91 uptakes was increased post irradiation were composed of mainly hypoxic cells. On the other hand, many viable areas were observed in tumors when the increase in ^{99m}Tc-HL91 uptake was relatively small. ***Conclusion:*** ^{99m}Tc-HL91 uptake in tumors did not always relate to their sensitivities to radiation therapy. Sequential ^{99m}Tc-HL91 imagings post irradiation showed that the increase in ^{99m}Tc-HL91 uptake in tumors predicted a poor response to radiation therapy, and that a decrease or no change suggested that radiation therapy would be effective. Monitoring by ^{99m}Tc-HL91 imaging is a good tool to predict the radiosentivities of tumors.

Key words: ^{99m}Tc-HL91, tumor hypoxia, radiation therapy, prediction, radiosensitivity

INTRODUCTION

OXYGENATION STATUS of tumor tissue is an important factor to determine the radiotherapeutic and chemotherapeutic effects,[1,2] and obtaining information on oxygen conditions of tumors before or during treatment may potentially predict the subsequent therapeutic efficacy. Tumor cell structure of circle-wise proliferation especially in the capillary vessel, which is called tumor code, is seen within the tumor.[3] Two possible mechanisms underlying the cause of intratumor hypoxia are considered.[4] One is that hypoxia is caused by the limited oxygen diffusion (100–180 μm) from the capillary vessel to the tissue, which occurs in two to three layers surrounding the tumor code. This is called diffusion-limited hypoxia, and hypoxic

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cells resulting from this mechanism are called chronically hypoxic cells. The other mechanism is the induction of acute hypoxic status by transient closure of tumor vessels. This hypoxic status caused by blood flow interruption is called perfusion-limited hypoxia, and hypoxic cells resulting from this mechanism are called acutely hypoxic cells.[5] Since Thomlinson et al. have shown that tumor code 160 μm or less in diameter is not associated with necrosis, but more than 200 μm-tumor code is always associated with central necrosis, oxygen concentration of cancer cells is known to be largely involved in radiosensitivity. The practical proportion of hypoxic cells was reported to vary widely, in experimentally induced tumors ranging from less than 1% to 80% or more, with a mean of approximately 15%.[6,7] In regard to the relationship between oxygenation condition and radiosensitivity, biological effect is larger in the presence of oxygen than in its absence, and oxygen enhancement ratio is 2.5 to 3.0 times higher in the presence than absence of oxygen (i.e., oxygen effect), when the radiation dose is high with a low LET radiation. Such changes in radiosensitivity occur in oxygen concentrations ranging between 0 and 30 mmHg. The intermediate sensitivity lying between anoxia and sufficient oxygen is caused by an oxygen partial pressure of 3 mmHg.[3]

It has already been reported that Tc-99m labeled 4,9-diaza-3,3,10,10-tetramethyldodecan-2,11-dione dioxime (^{99m}Tc-HL91) is a tumor hypoxic marker.[8–12] In this study, we investigated the relationship between ^{99m}Tc-HL91 uptake in tumors and tumor response to radiation in athymic mice bearing human tumors to determine whether ^{99m}Tc-HL91 is useful in predicting radiotherapeutic efficacy in clinical settings.

MATERIALS AND METHODS

Animal models

Athymic mice implanted with human tumors (the human gastric tumor cell line MKN45, the human epidermoid carcinoma cell line KB-31, and the human lung adenocarcinoma cell line HLC) were used. Tumor cells of 1×10^7 were subcutaneously inoculated into the thighs of mice, and when tumor cells grew macroscopically (300–600 mm^3), they were used for the experiment. In a KB-31 irradiation study with 20 Gy, smaller sized tumors (100–200 mm^3) were also prepared as experimental material.

^{99m}Tc-labeled HL91 for scintigraphy

HL91 was labeled with ^{99m}Tc and scintigraphy was conducted as described in our previous paper. Briefly mice were injected intravenously with 0.1–0.2 m*l* (37–74 MBq) of ^{99m}Tc-HL91. Imaging was carried out 3 hours later. Scintigraphy with ^{99m}Tc-HL91 was performed just before irradiation and at the end of the study. In a study of KB-31 irradiated with 20 Gy, scintigraphy was also performed on post-irradiation days 4 and 8 in addition to before irradiation and at the end of study.

Irradiation

Mice were intraperitoneally anesthetized with pentobarbital sodium and immobilized with a fixed apparatus, and the thigh inoculated with tumor cells was irradiated at a dose rate of 150 cGy/min with a 4 MV linear accelerator (Mitsubishi ML6M). A bolus material equivalent to water, 5 mm in thickness (Floatation Bed Pad: Sakura IRYOKI) was placed on the leg to be irradiated. Animals were allocated to six irradiation groups (HLC with 10 Gy, KB-31 with 10 Gy, MKN45 with 15 Gy, MKN45 with 20 Gy, KB-31 with 20 Gy [large tumors], KB-31 with 20 Gy [small tumors]) of 2 to 4 each. All values are single radiation doses. Post-irradiation follow-up was performed during 28 days in HLC with 10 Gy and KB-31 with 10 Gy, 19 days in MKN45 with 15 Gy, 21 days in MKN45 with 20 Gy, 16 days in KB-31 with 20 Gy (large tumors) and 14 days in KB-31 with 20 Gy (small tumors). The observation periods were different in each group because the timing to sacrifice mice was determined by the condition of the mice. In each irradiation group, 2 to 4 non-irradiated animals served as a control. Tumor diameters in 3 directions, length (L), width (W) and height (H), were measured every 2 or 3 days over a 2- to 4-week period after irradiation in all animals, including non-irradiated animals, to evaluate tumor growth. Tumor volumes (mm^3) were calculated on the assumption that the tumors were hemi ellipsoids where volume equals $4\pi/3 \cdot L/2 \cdot W/2 \cdot H/2$, that is, approx. 0.5LWH.[13,14]

Uptake of ^{99m}Tc-HL91

Regions of interest (ROI) were defined in the tumor and the whole body on scintigrams to determine the uptake of ^{99m}Tc-HL91. The accumulation ratio of tumor to whole body (T/WB) was calculated. At end of the study, blood, muscle, and tumor were obtained to determine the tracer uptake. The weight and radioactivity of the blood, muscle, and tumor were measured. The uptake of radioactivity in

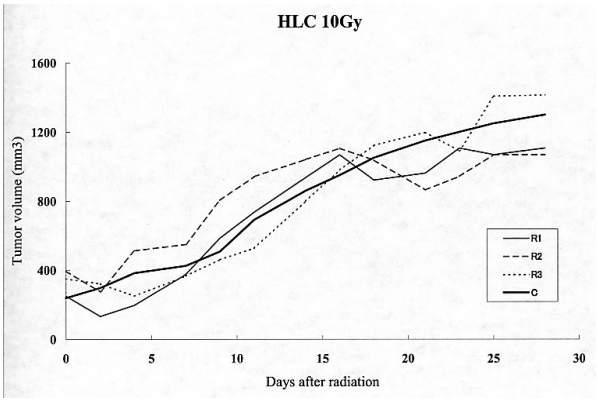

Fig. 1 Growth curves of lung adenocarcinoma (HLC). R1–R3: 10 Gy-irradiated tumors, C: non-irradiated tumor

the blood, muscle and tumor (% injected dose/g tissue [%ID/g]) was obtained, and the tumor-to-blood (T/B) ratio was also calculated.

Distribution of intratumor ^{99m}Tc-HL91

Radioactivity distribution within the tumor was assessed using autoradiograms and hematoxylin-eosin (HE) staining of tumor sections, as described in our previous paper.[12] Some mice were sacrificed 4 hours after injection of ^{99m}Tc-HL91 followed by autoradiography.

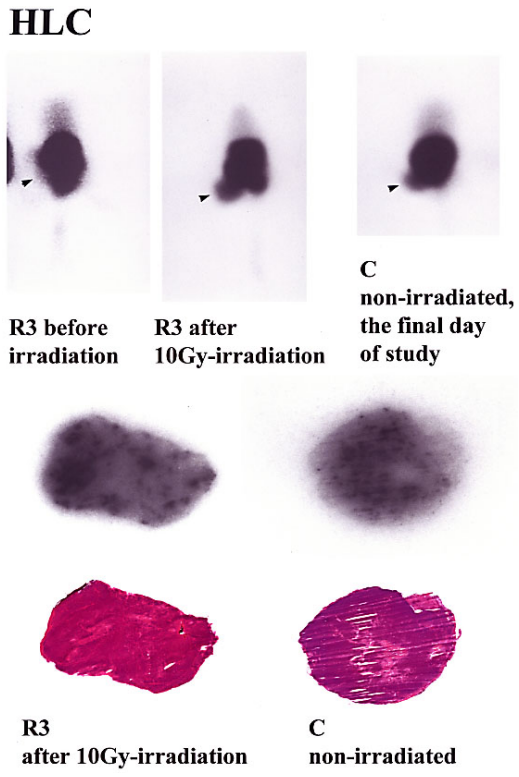

Fig. 2 Scintigrams of HLC tumors (R3 and C) and autoradiograms and HE staining of both tumor sections.

RESULTS

Irradiation of the lung adenocarcinoma HLC × 10 Gy

Figure 1 shows growth curves of HLC. R1, R2, and R3 are tumors exposed to 10 Gy; C is average of non-irradiated tumors. Tumor growth was monitored over a 4-week period. All of the irradiated tumors grew as well as non-irradiated tumor. The lung adenocarcinoma HLC hardly responded to radiation. The T/B ratio at the end of study (post-irradiation day 28) was 1.8 for R1, 1.7 for R2, 1.9 for R3, and 1.7 for C, revealing that the uptake of ^{99m}Tc-HL91 in the irradiated group did not differ from that in the non-irradiated tumors. Tracer uptake in all tumors in the irradiated group was higher at the end of study than before irradiation, as evidenced by scintigraphy (T/WB: 1.1% vs. 3.3% for R1, 1.2% vs. 3.7% for R2, and 1.3% vs. 8.0% for R3).

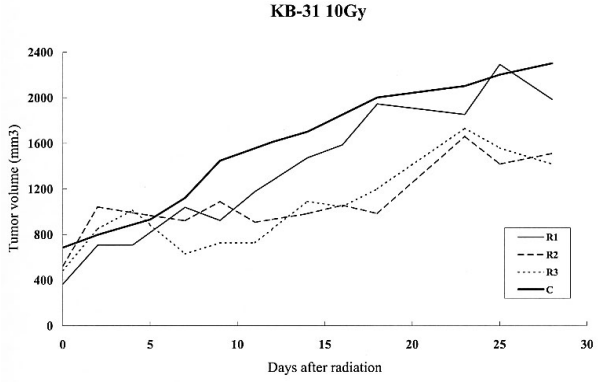

Fig. 3 Growth curves of epidermoid carcinoma (KB-31). R1–R3: 10 Gy-irradiated tumors, C: average of non-irradiated tumors

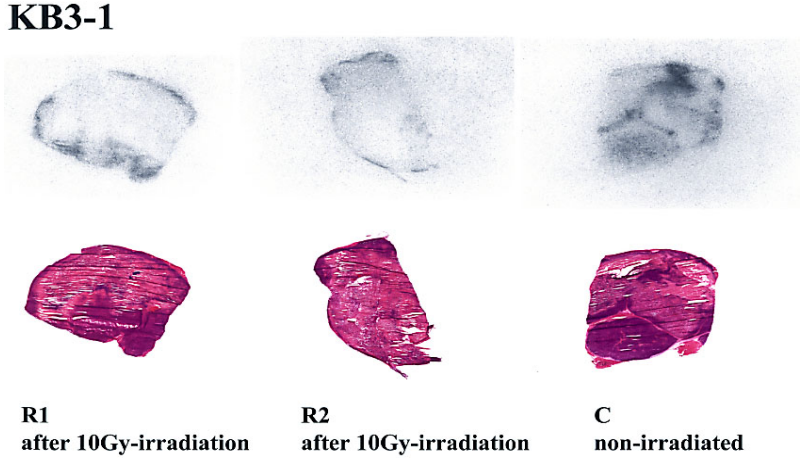

Fig. 4 Autoradiograms and HE staining of KB-31 tumor sections (R1, R2 and C). ^{99m}Tc-HL91 was strongly accumulated in the border zone.

Figure 2 shows scintigrams, autoradiograms, and HE staining of tumor sections in irradiated tumor R3 and non-irradiated tumor C. As found in scintigraphic findings, tracer uptake for R3 also visually increased at the end of study as compared with that before irradiation. Similar uptake was seen in non-irradiated tumor. Both autoradiograms and HE staining of tumor sections indicated low uptake of ^{99m}Tc-HL91 in viable and necrotic areas and an avid tracer uptake in the border zone suggesting a hypoxic region.

Irradiation of the epidermoid carcinoma KB-31 × 10 Gy

Growth curves of the epidermoid carcinoma KB-31 are given in Figure 3. Growth rate was delayed in irradiated tumors R2 and R3, as compared with non-irradiated tumors. On the other hand, tumor growth in irradiated tumor R1 was not inhibited. The T/B ratio of ^{99m}Tc-HL91 in the irradiated group (0.7 for R1, 0.4 for R2, and 0.5 for R3) was lower than that in the non-irradiated tumors (1.7) at the end of the study (post-irradiation day 28).

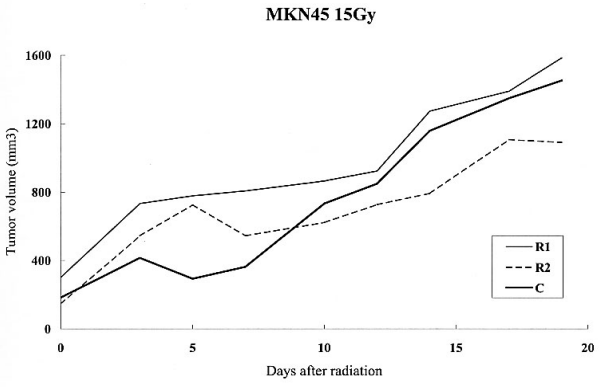

Fig. 5 Growth curves of gastric cancer (MKN45).
R1–R2: 15 Gy-irradiated tumors, C: average of non-irradiated tumors

Figure 4 shows autoradiograms and HE staining of tumor sections in irradiated tumors R1 and R2 and non-irradiated tumor C. Autoradiography revealed a partial accumulation of ^{99m}Tc-HL91 in the lower part of the tumor in irradiated tumor R1, and generally little accumulation in irradiated tumor R2. The accumulation of ^{99m}Tc-HL91 was relatively noticeable in non-irradiated tumor. An area where ^{99m}Tc-HL91 was not accumulated corresponded to the histologically necrotic area. Low uptake of ^{99m}Tc-HL91 in irradiated tumors may have been reflected by uptake defect in necrotic areas that occurred as a result of irradiation.

When ^{99m}Tc-HL91 uptake was compared in the ROI, placed on scintigrams, before irradiation and at the end of study, tumor uptake decreased in tumors whose growth was inhibited by radiation (T/WB: 7.3% vs. 3.6% for R2 and 1.5% vs. 1.2% for R3). In irradiated tumor R1 in which tumor-growth inhibition was less than that in the other two irradiated tumors, T/WB increased from 1.7% to 2.7%.

The uptake of radioactivity before irradiation was almost the same in irradiated tumors R1 (T/WB: 1.7%) and R3 (T/WB: 1.5%), while it was much higher in irradiated tumor R2 (T/WB: 7.3%) than in these two tumors. Tumor response to radiation was markedly poorest in irradiated tumor R1. There was no correlation between pre-irradiation uptake of ^{99m}Tc-HL91 and tumor response to radiation.

Irradiation of the gastric cancer MKN45 × 15 Gy

Figure 5 shows growth curves of MKN45 in 15 Gy-irradiated tumors R1 and R2 and non-irradiated tumors C. Tumor growth was not inhibited in irradiated tumor R1, as

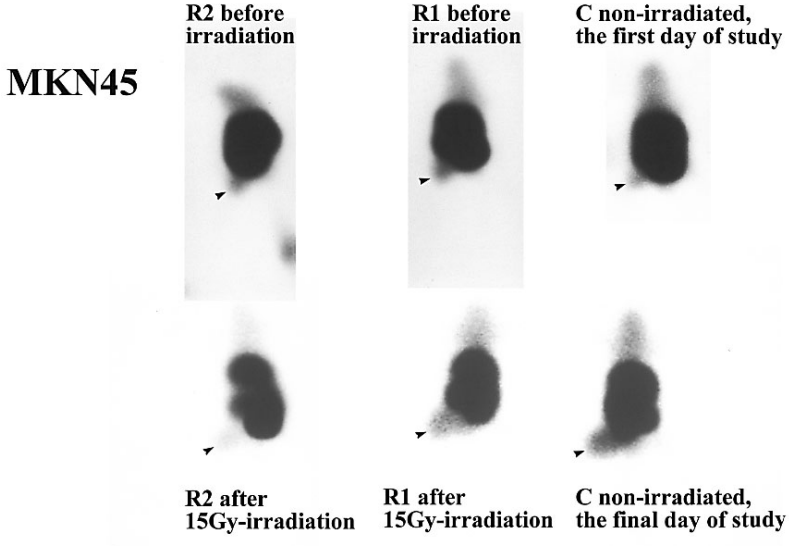

Fig. 6 Scintigrams of MKN45 tumors (R1, R2 and C).

well as non-irradiated tumor, but inhibited in irradiated tumor R2. A decreased uptake of radioactivity for R2 and a slightly increased uptake for R1 were seen on scintigrams (Fig. 6). Tracer uptake apparently increased in non-irradiated tumors. The T/B ratio at the end of the study (post-irradiation day 19) was lower in irradiated tumor R2 (0.68) than non-irradiated tumors (0.95), but did not greatly differ in irradiated tumor R1 (0.82) from the non-irradiated tumors. When tumor uptake was compared in the ROI before irradiation and at the end of the study (post-irradiation day 19), T/WB decreased from 2.6% to 2.0% for R2 that showed tumor-growth inhibition. In irradiated tumor R1 whose growth was not inhibited, however, tumor uptake (T/WB) increased from 3.1% to 4.3%.

Irradiation of the gastric cancer MKN45 × 20 Gy

Figure 7 shows growth curves of the gastric cancer MKN45 for R1, R2, and R3 and C. Tumor growth was inhibited in irradiated tumors, as compared with the non-irradiated tumors. Scintigraphy visually revealed that the increase in tracer uptake was less in irradiated tumor R2 than in non-irradiated tumors (Fig. 8). Tumor uptake greatly increased at the end of study (post-irradiation day 21) in the non-irradiated tumors, as indicated by T/WB (0.8% vs. 3.7%). In irradiated tumors, on the other hand, tumor uptake only slightly increased at the end of study, as compared with before irradiation (T/WB: from 0.7% to 1.1% for R1, 1.6% to 2.3% for R2, and 0.5% to 1.5% for R3). Tumor uptake increased by 2.9% (3.7–0.8%) at the end of study, as compared with before irradiation, in the non-irradiated tumors; however, among the irradiated tumors, tumor R3 exhibited an at most 1.0% (1.5–0.5%) increase in tumor uptake.

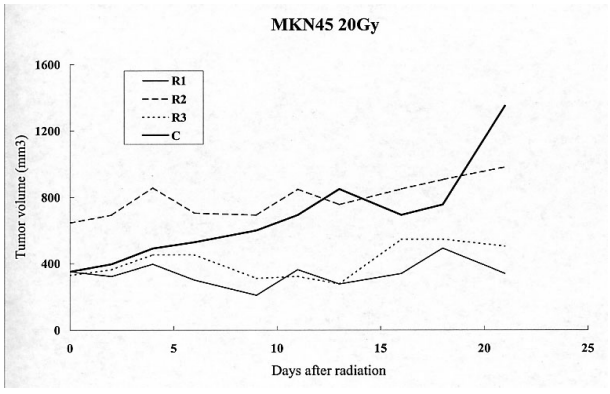

Fig. 7 Growth curves of gastric cancer (MKN45).
R1–R3: 20 Gy-irradiated tumors, C: average of non-irradiated tumors

Irradiation of the epidermoid carcinoma KB-31 × 20 Gy

In a KB-31 irradiation study with 20 Gy, tumors were divided into a small group (100–200 mm^3) and large group (300–600 mm^3), because it was expected that smaller tumors than those used in the above four irradiation studies could be favorably reduced by irradiation and thus the correlation of tumor reduction with ^{99m}Tc-HL91 uptake was made clearer. Furthermore, to predict radiotherapeutic effects more precisely, tumor uptake was assessed by performing scintigraphy four times (before irradiation, 4 and 8 days after irradiation, and at the end of the study).

Figure 9 shows growth curves in 20 Gy-irradiated large tumors (similar in size to that in the 10 Gy-irradiation study). Tumor uptake gradually increased with increasing tumor size in non-irradiated tumor (T/WB: 1.7%, 2.4%, 2.7% and 3.6% before irradiation, 4, 8 and 16 days after irradiation respectively). In irradiated tumors R1 (T/WB: 3.7%, 2.2%, 2.8% and 3.3% before irradiation, 4, 8 and 16 days after irradiation respectively) and R2 (T/WB: 2.7%, 2.3%, 3.0% and 4.2% before irradiation, 4, 8 and 16 days after irradiation respectively) of which growth was not inhibited, tumor uptake decreased on post-irradiation day 4 but increased on day 8 as compared with before irradiation. Tumor uptake at the end of the study was slightly less than that before irradiation in irradiated tumor R1. In

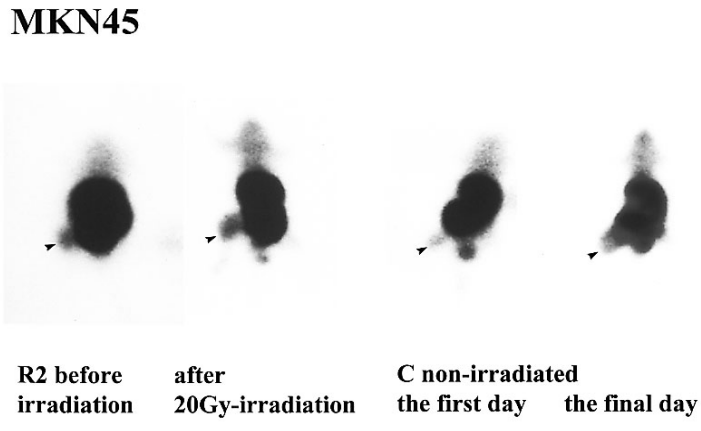

Fig. 8 Scintigrams of MKN45 tumors (R2 and C).

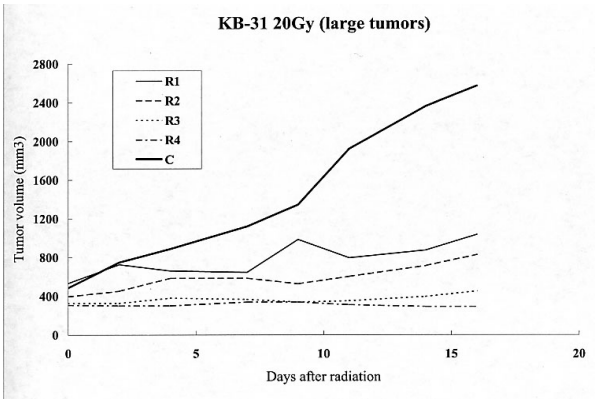

Fig. 9 Growth curves of large tumors (300–600 mm^3) of epidermoid carcinoma (KB-31).
R1–R4: 20 Gy-irradiated tumors, C: average of non-irradiated tumors

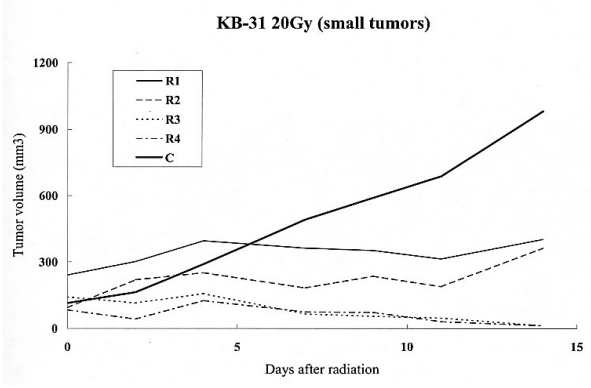

Fig. 10 Growth curves of small tumors (100–200 mm^3) of epidermoid carcinoma (KB-31).
R1–R4: 20 Gy-irradiated tumors, C: average of non-irradiated tumors

irradiated tumor R2 in which tumor growth was slight, tumor uptake increased at the end of the study. On the other hand, in irradiated tumors R3 (T/WB: 3.3%, 1.9%, 1.8% and 1.9% before irradiation, 4, 8 and 16 days after irradiation respectively) and R4 (T/WB: 3.4%, 2.4%, 2.1% and 1.7% before irradiation, 4, 8 and 16 days after irradiation respectively) of which growth was inhibited, tumor uptake plateaued for R3 or gradually decreased for R4.

Tumor sections were divided into 9 segments in each of the irradiated tumors. Each segment was microscopically assessed to identify viable, hypoxic or necrotic status. Five segments were viable in R1, while R2 consisted of 6 border-zone segments (hypoxic). Five necrotic segments were observed in R3 and R4. This means that R1 tumor was mainly composed of viable cells, R2 tumors of mixed necrotic and viable cells (border-zone), and R3 and R4 tumors of necrotic tumors.

Figure 10 shows growth curves of small tumors exposed to 20 Gy. In a similar manner to large tumors, tumor uptake increased with increasing tumor size in non-irradiated tumors (T/WB: 1.2%, 2.6%, 2.7% and 3.9% before irradiation, 4, 8 and 14 days after irradiation respectively). In irradiated tumors R1 and R2, tumor growth was inhibited a little. In tumor R1 whose growth was comparatively inhibited (tumor size plateaued), tumor uptake transiently decreased on post-irradiation day 4 but increased on day 8, with the increase marked at the end of the study (T/WB: 3.1%, 2.5%, 3.4% and 5.3% before irradiation, 4, 8 and 14 days after irradiation respectively). In tumor R2 in which growth inhibition was minimal (tumor size slightly increased), tumor uptake increased a little (T/WB: 1.4%, 1.4%, 1.5% and 1.9% before irradiation, 4, 8 and 14 days after irradiation respectively). Tumors R3 and R4 exhibited a marked growth inhibition after irradiation, and viable tumor cells were detected only in a part of HE staining of the tumor sections removed at the end of the study. In these tumors, tumor uptake gradually decreased (T/WB: 2.3%, 2.0%, 1.6% and 1.0% for R3, 2.2%, 1.4%, 1.2% and 1.0% for R4, before irradiation, 4, 8 and 14 days after irradiation respectively). Little viable tumor tissue existed and the majority of the sections consisted muscle and necrosis in R3 and R4. When tumor sections were divided into 9 segments for histological assessment, border-zones of necrotic and viable area were seen in 6 of the 9 segments for R1, viable areas in 6 of the 9 segments for R2, and necrotic areas in 7 and 9 of the 9 segments for R3 and R4 respectively. That is, R1 tumor was mainly composed of mixed necrotic and viable tissues (border-zone) and R2 tumors of viable tissues. In tumors R3 and R4, the majority of the tumor was necrotic, although some parts remained viable.

DISCUSSION

Assessment of radiosensitivity of cancer before or during radiation therapy is an urgent issue, and non-invasive methods should be developed to understand oxygen conditions of tumors clinically. Since misonidazole was discovered as a hypoxic cell sensitizer about 20 years ago, several hypoxic cell markers have been developed. Chapman et al. reviewed the measurement of oxygen status of tumors and tumor radioresistance by a radionuclide imaging technique.[15] ^{201}Tl has also been investigated as a marker to predict radiosensitivity. A low uptake of ^{201}Tl has been shown to reflect the presence of non-viable cells, but it remains undetermined whether tumor cells are viable or hypoxic when ^{201}Tl is somewhat taken up by these cells. Fukumoto et al. have also posed a question as to the usefulness of ^{201}Tl in predicting the radioresistance of lung cancer.[16]

Our previous study showed that ^{99m}Tc-HL91 was accumulated significantly more in hypoxic areas than in necrotic or viable ones. We have also demonstrated that uptake of ^{99m}Tc-HL91 was less in necrotic areas than in

viable ones so that it could distinguish oxidation status in tumor tissues. The tumor-to-blood ratio of ^{99m}Tc-HL91 in the tumors we studied previously was higher than that of other ^{99m}Tc-labeled hypoxic markers, such as ^{99m}Tc-BMS181321[17] and ^{99m}Tc-BRU59-21.[18] Iodine-123-labeled iodoazomycin arabinoside (IAZA) is also reported to be a hypoxic marker,[19,20] but it is unlikely to have any advantages over ^{99m}Tc-HL91.

The following findings were drawn from this experimental study by use of ^{99m}Tc-HL91:

1. Accumulation of ^{99m}Tc-HL91 in tumors was not capable of predicting the subsequent tumor response to radiation, except for tumors such as lung adenocarcinoma, HLC, where radioactivity was always avidly taken up with a poor response to radiation.
2. For tumors where ^{99m}Tc-HL91 uptake was increased post irradiation, growth was not inhibited, meaning their response to radiation was poor. Autoradiography showed the tumor tissue was composed of mainly hypoxic cells, especially when uptake was highly increased.
3. Tumor growth was inhibited post radiation, which was accompanied by decreased tracer uptake in tumors. Autoradiography indicated that the majority of tumor tissues were necrotic.
4. Serial ^{99m}Tc-HL91 imaging showed that a small increase in ^{99m}Tc-HL91 uptake suggested a subsequent poor response to irradiation even though their growth was delayed transiently. Autoradiography showed that such tumor tissues still possessed numerous viable cells.

Hypoxic areas in tumor tissue increase in size in parallel with tumor growth, since blood flow fails to provide sufficient oxygen. When the tumor does not respond to radiation, it keeps growing and then, its oxygen status becomes poor. After irradiation of the tumor, its oxygen status is changed in two ways: to a mixture of hypoxic and viable cells or one of hypoxic and necrotic ones. It is not certain what underlies this difference in the response to irradiation, but our present study indicated these hypothetical ways by serial changes in the ^{99m}Tc-HL91 uptake post radiation. Namely, in the radiosensitive tumors we studied, their accumulation of ^{99m}Tc-HL91 gradually decreased with reduced tumor size and increase in necrotic areas. On the contrary, in tumors with a relatively poor response to irradiation, ^{99m}Tc-HL91 uptake transiently decreased immediately after radiation, and then increased, probably because viable cells persisted there. Furthermore, in tumors with no response to irradiation, we observed significant increase in ^{99m}Tc-HL91 uptake, probably because tumor tissues were composed mainly of hypoxic cells.

We speculate about the clinical use of ^{99m}Tc-HL91 imaging as follows;

(1) When uptake of ^{99m}Tc-HL91 is decreased constantly post radiation, the tumor would be radiosensitive.

(2) When uptake of ^{99m}Tc-HL91 is slightly or transiently decreased, the response to radiation would be inadequate, and then, repeated irradiation may be of value.

(3) When uptake of ^{99m}Tc-HL91 is significantly increased, the tumor would be resistant to radiation, and therefore, additional irradiation is unlikely to be effective.

Further studies are needed to clarify the relation between radiation effect and hypoxic status under different conditions.

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