Tracer Feasibility for Monitoring Tumor Radiotherapy: A Quadruple Tracer Study with Fluorine-18-Fluorodeoxyglucose or Fluorine-18-Fluorodeoxyuridine, L-[Methyl-^{14}C]Methionine, [6-^{3}H]Thymidine, and Gallium-67

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In a rat AH109A tumor model, metabolic tracers for glucose, amino acid, and nucleic acid metabolisms [2-deoxy-2-[^{18}F] fluoro-D-glucose (^{18}FDG), L-[methyl-^{14}C]methionine (^{14}C-Met), [6-^{3}H]thymidine (^{3}H-Thd), and 2′-deoxy-5-[^{18}F]fluorouridine (^{18}FdUrd)], and the conventional radionuclide ^{67}Ga-citrate were used to assess the feasibility of monitoring tumor radiotherapy using a quadruple tracer technique. Two combinations of four tracers (^{18}FDG or ^{18}FdUrd, ^{14}C-Met, ^{3}H-Thd and ^{67}Ga) were compared in a time-course study after single-dose irradiation (20 Gy) and were also used in a dose-dependency study performed 6 days after 5, 10, 15, or 20 Gy of irradiation. Fluorine-18-FDG showed a large change in uptake and a steady response to radiotherapy. Fluorodeoxyuridine showed a rapid decrease after radiotherapy, but the range of change in uptake was narrow. Gallium-67 could not detect tumor response early after treatment, but showed a marked change in uptake later. [6-^{3}H]Thd and ^{14}C-Met showed a rapid response to irradiation and a high sensitivity for monitoring radiotherapy, suggesting that they may be feasible for PET studies.

J Nucl Med 1991; 32:2118–2123

Tumor size measurement by x-ray or CT scan has been the standard method of treatment evaluation in patients undergoing radiotherapy and chemotherapy (*1*). However differentiation of the residual viable tumor tissue from areas of necrosis or fibrosis (*2*) and predicting the outcome during treatment (*3*) are both difficult with such imaging methods. Various in vitro techniques for predicting the probability of tumor control have been reported (*4*), but they all require tumor biopsy to be performed.

Positron emission tomography (PET) using metabolic tracers can noninvasively demonstrate various biologic properties of tumors, including differentiating benign and malignant disease (*5–8*), determining the grade of malignancy (*9–12*), and assessing prognosis (*13*). It is expected to become a valuable method of treatment evaluation (*14–16*). Characterization of the behavior of various metabolic tracers in different tumor conditions is necessary to assess the ability of each tracer to monitor the response to treatment. In our previous study (*15*), L-[methyl-^{11}C]methionine (^{11}C-Met) uptake by the AH109A tumor showed a rapid decrease after 20 Gy of irradiation and was followed by necrosis and progressive tumor shrinkage. Thus, ^{11}C-Met uptake was suggested to sensitively indicate biologic response of the tumor to radiotherapy. In this study, PET tracers that can follow glucose, amino acid, and nucleic acid metabolism as well as the conventional tracer ^{67}Ga-citrate were compared in the same rat tumor model to determine the feasibility of monitoring tumor radiotherapy with a quadruple tracer technique.

MATERIALS AND METHODS

Radiopharmaceuticals

2-Deoxy-2-[^{18}F]fluoro-D-glucose (^{18}FDG) and 2′-deoxy-5-[^{18}F]fluorouridine (^{18}FdUrd) were synthesized using an automated system (*17,18*). Radiochemical purity of both tracers was over 99%. The following tracers were purchased commercially: ^{67}Ga (Nihon Medi-Physics, over 99% of radiochemical purity), ^{14}C-Met (as a substitute for ^{11}C-Met, Amersham International, specific activity: 2.07 GBq/mmol), and [6-^{3}H]thymidine (^{3}H-Thd; as a substitute for ^{11}C-Thd, Amersham International, specific activity: 925 GBq/mmol).

Tumor and ^{60}Co Irradiation

A 0.1 ml suspension of 7×10^{6} AH109A cells was subcutaneously inoculated in the thighs of young male Donryu rats weigh-

Received Dec. 19, 1990; revision accepted Apr. 23, 1991.

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ing 160–200 g. Irradiation was performed when the tumors grew to between 1.0 and 1.5 cm in diameter. Rats were anesthetized and the tumors were exposed to a single dose of ^{60}Co irradiation at a dose rate of 1.04 Gy/min using a copper aluminum filter (*15*). Nonirradiated tumors in rats handled in the same manner, including anesthesia, were used as controls.

Growth Curve

Tumors were measured with vernier calipers; the product of the three principal diameters of each tumor was designated as the "tumor volume," and tumor growth curves were drawn as described previously (*19*). Four groups of eight rats received irradiation (5, 10, 15, or 20 Gy) and a group of nonirradiated controls were also used in the tumor growth study.

Quadruple Tracer Study

Two combinations of four tracers each (^{18}FDG or ^{18}FdUrd, ^{14}C-Met, ^{3}H-Thd, and ^{67}Ga) were used to compare these five tracers. A dose of 2.5 μCi (92.5 kBq) of ^{67}Ga in 0.25 ml of saline was injected through a lateral tail vein. Twenty-four hours later, a mixture of three other tracers [30 μCi (1.11 MBq) of either ^{18}FDG or ^{18}FdUrd and 6 μCi (222 kBq) each of ^{14}C-Met and ^{3}H-Thd] in 0.25 ml of saline was injected, and the rats were killed 30 min later. There was no significant residual tracer at the injection site. Tissue samples were excised and weighed, and the radioactivity was measured. The whole tumor was sampled in most of the rats, but when the tumor was too large, a cross-sectional slice at its maximum diameter was sampled. For the time-course study, the ^{18}FDG tracer combination was administered to four groups of 12–19 rats at 1, 2, 3, and 6 days after irradiation (20 Gy) and also to a control group. Other groups of 7–13 rats were administered the ^{18}FdUrd tracer combination. For the dose-response study, the ^{18}FDG and ^{18}FdUrd tracer combinations were each administered to four groups of seven rats 6 days after irradiation (5, 10, 15, and 20 Gy) and were also given to control groups (Table 1). Tumors at 6 days after 10 and 20 Gy of irradiation were processed for microscopic observation with hematoxylin and eosin staining.

Radioactivity Measurement

Fluorine-18 radioactivity was counted just after tumor sampling using an automated gamma-counter with a 450–600 keV window (spillover from ^{67}Ga was 0.133%). At this time, accurate ^{67}Ga counting with the 50–450 keV window was difficult because spillover from ^{18}F was 70.8%. Twenty-four hours after the experiment, ^{18}F ($T_{½}$ = 109.7 min) had decayed and ^{67}Ga could be counted without contamination. When liquid scintillation counting of ^{3}H and ^{14}C was performed at this time, significant contamination from ^{67}Ga was observed, probably because of the high photon flux from the low-energy gamma rays of ^{67}Ga. Accordingly, 33 days later when ^{67}Ga ($T_{½}$ = 3.2 day) had decayed, the tumor tissue samples were digested and bleached with perchloric acid and hydrogen peroxide (1:3) in a heater at 80°C. Samples were then mixed with the scintillation cocktail and left to stand at room temperature overnight, after which ^{3}H and ^{14}C were measured using a liquid scintillation counter with the double-window technique. Calibration for quenching (channel ratio method) and computation of the radioactivity of each nuclide were performed using the computer program incorporated in the counter. The contamination of ^{3}H with ^{14}C was 1.3%, and that of ^{14}C with ^{3}H was 0.35% (Table 2). Tissue radioactivity was expressed as the differential uptake ratio (DUR) (*15*):

$$\text{DUR} = \frac{\text{tissue counts/tissue weight}}{\text{injected dose counts/body weight}}.$$

TABLE 1
Time-Line Diagram Describing the Sequence of the Studies

Days −7 or −8	Subcutaneous injection of AH109A tumor cells
Day 0	^{60}Co irradiation*
Day n − 1	Intravenous injection of ^{67}Ga (2.5 μCi in 0.25 ml)
Day n*	Intravenous injection of a mixture of (^{18}FDG+^{14}C-Met+^{3}H-Thd) in 0.25 ml or (^{18}FdUrd+^{14}C-Met+^{3}H-Thd) in 0.25 ml
	Rats were killed 30 min later. Tissue sampling and ^{18}F measurement by γ-counter (450–600 keV)
Day n + 1	Gallium-67 measurement by γ-counter (50–450 keV)
	Samples stored at 4°C for the decay of ^{67}Ga
Day n + 33	Sample repreparation for β-counting with a LSC
	^{3}H (0–400 keV) and ^{14}C (400–670 keV) measurements by LSC

* Irradiation dose and the number of days after irradiation for the studies.

	Dose	Day n
Time-course study groups	20 Gy	1, 2, 3, 6
Dose-responsive study groups	5, 10, 15, 20 Gy	6

Each study had nonirradiated control groups.
LSC = Liquid scintillation counter.

RESULTS

Dose-response curves for tumor growth are shown in Figure 1. Each curve gives the mean and standard deviation of the data from eight tumors. After 5 Gy of irradiation, tumors showed slight shrinkage followed by rapid regrowth. Doses of 10, 15, and 20 Gy produced a similar response of tumor swelling on 1 day after irradiation (Day 1) followed by shrinkage until Day 6. Regrowth was seen on Day 7 after 10 Gy of irradiation ($p < 0.05$) and on Day 10 after 15 Gy ($p < 0.05$). No tumor regrowth was observed after a dose of 20 Gy. Instead, tumor shrinkage progressed until the lesion disappeared completely (data not shown).

Tumor uptake of the five tracers was compared after a single dose of 20 Gy irradiation (Fig. 2). This time-course study was continued for 6 days until tumor uptake of ^{11}C-Met was reduced to that for muscle (*15*). In nonirradiated control tumors, uptake of ^{67}Ga and ^{18}FDG was equal and the highest. Thymidine and methionine uptake was also equal and lower than ^{18}FDG uptake, while ^{18}FdUrd uptake was the lowest. After 20 Gy of irradiation, ^{3}H-Thd and ^{14}C-Met uptake decreased rapidly on Day 1 to 44.2% ± 11.7% and 52.5% ± 19.0% of the control level. A gradual and constant decrease in ^{18}FDG uptake became significant

TABLE 2
Contamination in the Differential Counting of Four Radionuclides

Nuclide	Window	Time after sampling and method of counting: 3 hr, γ-Counter	24 hr, γ-Counter	24 hr, LSC	33 days, LSC
^{18}F	450–600 keV ($T_{½}$:109 min)	0.13% with ^{67}Ga ∿∿∿	ND[1]		
^{67}Ga	50–450 keV ($T_{½}$:3.2 day)	71% with ^{18}F	0% with ^{18}F ∿∿∿	NA	ND[2]
^{3}H	0–400 keV			63% with ^{67}Ga	1.3% dpm with ^{14}C ∿∿∿
^{14}C	400–670 keV			33% with ^{67}Ga	0.35% dpm with ^{3}H ∿∿∿

LSC = liquid scintillation counter; ND[1] = not detectable (decayed to 0.0001%); ND[2] = not detectable (decayed to 0.001%); NA = not applicable (countable but low counting efficiency); and ∿∿= applied for differential counting.

when compared to the control on Days 2 and 3 (72.9% ± 13.1% and 62.8% ± 13.7% of the control level, respectively). Gallium-67 uptake showed a slight increase on Day 1, followed by a rapid decrease that became significant with respect to the control on Day 3 (53.7% ± 8.6% of the control level). Fluorodeoxyuridine uptake showed a steady decrease that became significant in comparison with the control on Days 1 and 2 (87.8% ± 12.6% and 52.7% ± 8.0% of the control level, respectively), although the DUR value range was very narrow [0.97 ± 0.14 (control) to 0.23 ± 0.13 (Day 6)]. A 50% decrease of tracer uptake was observed on Day 1 for ^{3}H-Thd and ^{14}C-Met, on Day 2 for $^{18}FdUrd$, and on Day 3 for ^{67}Ga and ^{18}FDG. The results of a micromorphometric study of the extent of necrosis in the AH109A tumors after 20 Gy of irradiation (*15*) are shown in Figure 3.

The dose-response study was performed on Day 6 (Fig. 4). Fluorodeoxyglucose and ^{67}Ga uptake by the tumors showed a similar pattern of irradiation dose-dependency. A significant decrease ($p < 0.005$) was observed between 5 and 10 Gy, but not between the control and 5 Gy for both ^{18}FDG and ^{67}Ga. Between 10 and 20 Gy, a significant difference was observed for ^{67}Ga uptake but not for ^{18}FDG. Methionine and thymidine showed another pattern of dose-dependent decrease. Significant differences ($p < 0.005$) were observed between the control and 5 Gy, 5 and 10 Gy, and 10 and 20 Gy, while no significant difference was observed between 10 and 15 Gy or 15 and 20 Gy. Fluorodeoxyuridine uptake showed significant differences between the control and 5 Gy ($p < 0.05$) as well as 5 and 10 Gy ($p < 0.001$), but not between 10 and 20 Gy.

Tumor growth rates and tracer uptake on Day 6 are compared in Table 3. The tumors with a high growth rate had a higher tracer uptake and those with regression had a lower uptake, but exact correlation was not observed. Photomicrographs of the tumors on Day 6 after 10 and 20 Gy of irradiation are shown in Figure 5. Both tumors were of the same volume (Fig. 1), but the intratumoral tissue components were different. The 10-Gy tumor showed multiple regenerative foci among the degenerating and necrotic tissue (Fig. 5A), while the 20 Gy tumor showed a large region of complete necrosis and a small area of degenerating tissue with a clear separating zone of infiltrating mononuclear and polymorphonuclear cells (Fig. 5B).

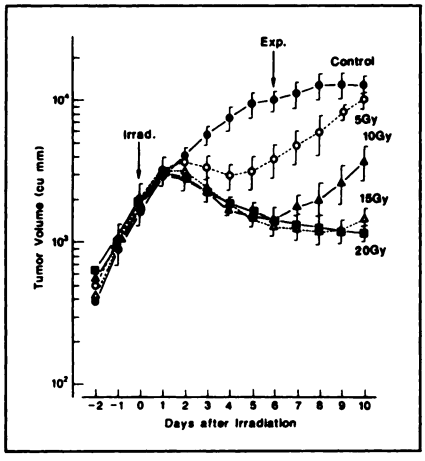

FIGURE 1. Tumor dose-response growth curves after ^{60}Co irradiation. AH109A solid tumors on the thighs of Donryu rats were irradiated on Day 0. The dose-response study was performed on Day 6. Each point shows the mean of eight tumors. ●: control, ○: 5 Gy, ▲: 10 Gy, △: 15 Gy and ■: 20 Gy.

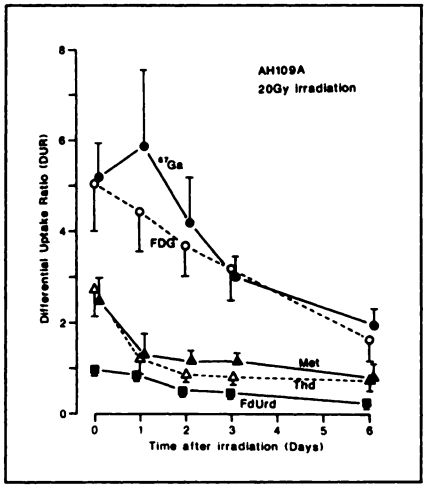

FIGURE 2. Changes of tumor uptake for five tracers after single-dose ^{60}Co irradiation (20 Gy). Two separate quadruple tracer uptake studies performed using AH109A tumors were averaged for each point. ●: ^{67}Ga, ○: ^{18}FDG, ▲: ^{14}C-Met, △: ^{3}H-Thd and ■: $^{18}FdUrd$.

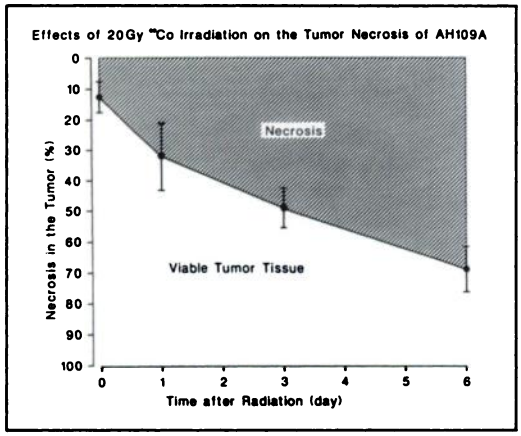

FIGURE 3. Micromorphometry of the necrotic tissue volume in AH109A tumors after ^{60}Co irradiation (20 Gy). (Reprinted by permission from Ref. 15.)

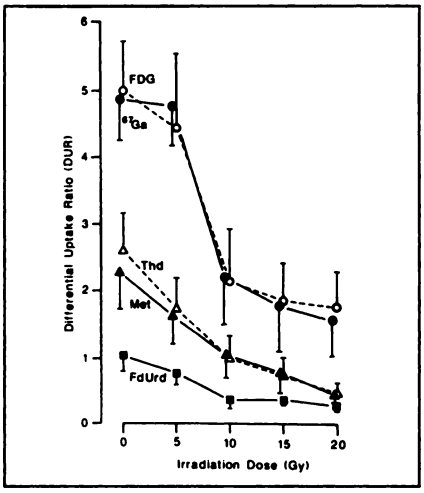

FIGURE 4. Dose-response curves for the tumor uptake of five tracers 6 days after single-dose ^{60}Co irradiation. Results for two separate quadruple tracer studies are shown. ●: ^{67}Ga, ○: ^{18}FDG, ▲: ^{14}C-Met, △: ^{3}H-Thd and ■: ^{18}FdUrd.

DISCUSSION

In this study, ^{3}H-Thd and ^{14}C-Met uptake by the tumors showed a rapid response to radiotherapy. Thymidine is rapidly incorporated into DNA in vivo in a manner that is independent of tissue perfusion (*20*) and has been used as a marker of cell proliferation (*11,21*). Thymidine uptake by experimental tumors shows a linear correlation to the tumor growth rate (*22*). The rapid reduction of ^{3}H-Thd uptake by irradiated tumors thus seems to result from damage to DNA synthesis and suggests the cessation of proliferation early after irradiation.

Fluorodeoxyuridine is incorporated into RNA synthesis and is also involved in the activity of thymidylate synthetase, the rate-determining enzyme of DNA synthesis (*23, 24*). Accordingly, the distribution of ^{3}H-Thd and ^{18}FdUrd in tumors is shown to be different by autoradiography (*23*). The distribution of ^{3}H-Thd and ^{3}H-deoxyuridine is also different, but both tracers are only observed in the viable cell zone and never in the necrotic region on autoradiography (*25*). In vitro, ^{3}H-Thd is incorporated into cells mainly in the exponential growth phase, but ^{14}C-uridine incorporation occurs in both the exponential and confluent phases (*26*). These findings suggest that ^{3}H-Thd is a marker of proliferating cells and ^{18}FdUrd is a marker of viable cells regardless of their proliferative status. In this study, changes in ^{18}FdUrd uptake were different from those for ^{3}H-Thd. The decrease of ^{18}FdUrd uptake may have represented the extension of tumor necrosis. Fluorodeoxyuridine uptake decreased faster than that of ^{18}FDG and ^{67}Ga after radiotherapy, but the change was within a narrow range. Although the value of ^{18}FdUrd in the diagnosis of brain tumors has been reported (*27*), such narrow uptake changes require sensitive detection, which is not advantageous when attempting to monitor tumor radiotherapy with PET.

Amino acid accumulation in tumor cells is mediated by active transport at the cell membrane (*28*) and by the increased metabolic demand of the cells themselves (*29*). In experimental tumors, more than 70% of the radioactivity was incorporated into the protein-bound fraction by 60 min and about 65% by 30 min after the injection of ^{14}C-Met (*30*). The rapid reduction of ^{14}C-Met uptake by tumors after irradiation seems to reflect both the inactivation of protein synthesis and damage to the membrane transport system. The same pattern of ^{3}H-Thd and ^{14}C-Met uptake after irradiation may suggest some linkage of amino acid metabolism to DNA synthesis in the process of radiation injury and repair.

After experimental radiotherapy, ^{18}FDG uptake was reduced in radiosensitive tumors but not in radioresistant tumors (*31*). AH109A is a radiosensitive tumor cell line and ^{18}FDG uptake by this tumor showed a gradual and constant decrease with time after irradiation. Fluorodeoxyuridine may be a useful tracer for PET monitoring of radiosensitive tumor response to radiotherapy because of the wide range of changes in its uptake. The process of radiation damage to glucose metabolism seems to be different from that affecting DNA and amino acid metabolism. Clinical ^{18}FDG studies monitoring radiochemotherapy of head and neck cancer (*14*), brain tumors (*32*), lung cancer (*33*), and liver tumors (*34*) have shown that the changes of ^{18}FDG uptake after tumor therapy correlated with the clinical evaluation of the effects of treatment.

The mechanism of ^{67}Ga accumulation in tumors has been studied since 1969 and various hypotheses have been presented, but its complexity still precludes general agreement (*35*). A large number of clinical studies have demonstrated its value for treatment evaluation, especially in malignant lymphoma (*36*). However, ^{67}Ga accumulation in inflammatory tissue and the presence of false-negative tumors are two known limitations (*37*). The slight increase of ^{67}Ga uptake by the tumors one day after irradiation in this study may be explained by its accumulation in the leukocytes and macrophages which were observed within and around the tumor after radiotherapy (*38*). This response was different from that of the metabolic tracers. Its

TABLE 3
Comparison of Tumor Growth Rate and Tracer Uptake 6 Days After Various Doses of Irradiation

Tumor	Volumetric tumor growth rate: Doubling time (days)	Volumetric tumor growth rate: Volume increase (%)	Tracer uptake (DUR) and % change: ^{18}FDG n = 7	^{18}FdUrd n = 7	^{67}Ga n = 12	^{14}C-Met n = 12	^{3}H-Thd n = 12
Control	1.21	+73%	4.99 ± 0.74 (100%)	1.04 ± 0.23 (100%)	4.87 ± 0.62 (100%)	2.28 ± 0.55 (100%)	2.59 ± 0.60 (100%)
5 Gy	3.26	+23%	4.44 ± 1.42* (89%)	0.78 ± 0.12† (75%)	4.78 ± 0.60* (98%)	1.62 ± 0.41‡ (71%)	1.74 ± 0.48‡ (67%)
10 Gy	7.61	+9%	2.14 ± 0.79‡ (43%)	0.39 ± 0.10§ (38%)	2.20 ± 0.70‡ (45%)	1.04 ± 0.34‡ (46%)	0.99 ± 0.38§ (38%)
15 Gy	−8.47	−8%	1.86 ± 0.59 (37%)	0.40 ± 0.03 (38%)	1.79 ± 0.65 (37%)	0.78 ± 0.32 (34%)	0.74 ± 0.29 (29%)
20 Gy	−6.67	−10%	1.78 ± 0.53* (36%)	0.33 ± 0.07* (32%)	1.57 ± 0.52† (32%)	0.48 ± 0.13§ (21%)	0.51 ± 0.16§ (20%)

Student's t-test was performed for comparisons between the control and 5 Gy, 5 and 10 Gy, and 10 and 20 Gy.
* Not significant.
† $p < 0.05$.
‡ $p < 0.005$.
§ $p < 0.001$.

uptake mechanism may not be metabolic, but the wide range of change in uptake later in the postirradiation period supports its value in monitoring the effects of radiotherapy.

The tumor growth depends on the balance between cell production and cell loss (*39*) and the tumor volume can be influenced by edematous swelling, the collapsing of dying tissues, or recurrent cell proliferation. Therefore, the volumetric tumor growth rate dose not directly reflect the tumor growth fraction, especially after therapeutic intervention. The histometric changes of necrotic and viable tumor tissue after radiotherapy are shown in Figure 3 (*15*). Nonirradiated AH109A tumors contained 12.6% ± 5.4% of necrotic tissue (87.4% viable). In the irradiated (20 Gy) tumors, the necrotic area was increased to 32.0% ± 11.3% (68.0% viable) on Day 1, to 49.0% ± 6.6% (51.0% viable) on Day 3, and to 68.6% ± 7.4% (31.4% viable) on Day 6 (n = 7–8, each). On the other hand, the irradiated tumor volume still remained at about 76% of the control tumor volume on Day 6. A comparison of tracer uptake with these data shows that ^{3}H-Thd and ^{14}C-Met uptake after 20 Gy of irradiation decreased before the extension of necrosis and the reduction of tumor volume occurred. Both ^{18}FdUrd and ^{18}FDG uptake decreased before tumor shrinkage occurred, but almost paralleled the extension of necrosis. To determine the histologic correlation with tracer uptake, further autoradiography studies are in progress.

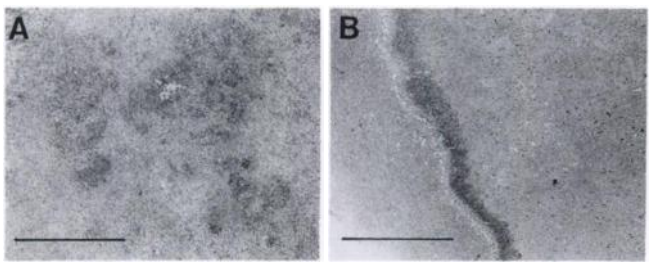

FIGURE 5. Photomicrographs of AH109A tumors 6 days after irradiation at 10 Gy (A) and 20 Gy (B). HE stain. Bar indicates 1 mm.

Tumors given 10 and 20 Gy of irradiation had the same volume on Day 6, but their tissue contents were clearly different as shown in Figure 5. The 10-Gy tumor had multiple foci of proliferation which represented the beginnings of recurrence, while the 20-Gy tumor had a large region of necrosis with a small area of degenerating cells and no signs of recurrence. Detection of these differences is very important when monitoring the effects of radiotherapy. Flurodeoxyglucose and ^{18}FdUrd could not detect differences between 10 Gy and 20 Gy of irradiation, while ^{14}C-Met, ^{3}H-Thd, and ^{67}Ga showed significant differences in uptake between these two irradiation doses. This suggests that ^{14}C-Met, ^{3}H-Thd, and ^{67}Ga may be more sensitive for the detection of early foci of recurrence than ^{18}FDG and ^{18}FdUrd.

CONCLUSION

We performed two separate quadruple tracer studies that demonstrated the relationships and responses of five tracers in the course of radiotherapy and obtained the following results:

1. Thymidine and ^{14}C-Met uptake showed a rapid and sensitive response to irradiation preceding both volumetric shrinkage and necrotic extension and detected early foci of recurrence.

2. Fluorodeoxyglucose uptake showed a wide range of change and a steady response to irradiation. It nearly paralleled the necrotic extension that precedes volumetric shrinkage.
3. Fluorodeoxyuridine showed a narrow range of change in uptake. Its response was similar to but occurred slightly earlier than that of ^{18}FDG.
4. Gallium-67 uptake did not change early after treatment, but showed a wide range of alteration later. Its uptake seemed not to be metabolically based, but it detected early foci of recurrence.

Our results suggest that thymidine and methionine, if labeled with ^{11}C, have a higher potential for rapid and sensitive PET monitoring of tumor radiotherapy. Our study was not able to demonstrate the mechanisms of metabolic damage by irradiation, but was able to demonstrate the feasibility of using tracers to monitor tumor radiotherapy.

ACKNOWLEDGMENTS

This work was supported by grant-in-aids (No. 0157058, 02152016, 02151072) for cancer research from the Ministry of Education, Science and Culture, Japan. The authors thank Mr. Y. Sugawara for photography and the staff members of the Cyclotron Radioisotope Center, Tohoku University.

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