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Detection of Hypoxic Cells in Murine Tumors Using the Comet Assay: Comparison with a Conventional Radiobiological Assay

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The comet (single-cell electrophoresis) assay has been developed as a method for measuring DNA damage in single cells after irradiation. We have developed our own methods and image analysis system for the comet assay to identify hypoxic fractions. *In vitro*, we tested our system using a cultured tumor cell line (SCCVII). *In vivo*, we compared the hypoxic fractions detected by this assay with those determined by the *in vivo-in vitro* clonogenic assay using two rodent tumors (SCCVII/C3H, EMT6/KU/balb/c), which exhibit different types of hypoxia: acute and chronic. *In vitro*, our method could differentiate hypoxic cells from oxic cells, using the parameter of tail moment. *In vivo*, there were good correlations between the hypoxic fractions determined by the comet assay and by the clonogenic assay, in SCCVII/C3H (r=0.85) and in EMT6/KU/balb/c (r=0.75) tumors. By comparison of the two methods in chronically hypoxic and acutely hypoxic tumors, we further confirmed that the comet assay is clinically useful for estimating hypoxic fractions of solid tumors.

Key words: Comet assay — Hypoxic cell fraction — Tumor — Chronically hypoxic cell — Acutely hypoxic cell

It is well known that the presence of hypoxic cells in solid tumors is an important cause of failure of radiation therapy and some chemotherapy regimens.[1)] Attempts to overcome this problem have included the use of bioreductive agents, such as tirapazamine, which are selectively toxic to hypoxic cells, heavy charged particle radiotherapy, and treatment with hyperbaric oxygen.

A previous study suggested that the hypoxic fraction differs with the tumor type.[2)] Radiobiological methods of measuring the hypoxic fractions in rodent tumors include the paired survival curve assay,[3, 4)] the clamped tumor control dose (TCD50) assay[5)] and the clamped tumor growth delay assay.[6)] However, they are not suitable for clinical use. There is no method for the routine measurement of the intratumoral hypoxic fraction in patients.

A comet assay (single-cell electrophoresis assay) has been developed for measuring the DNA damage after genotoxic treatment. It is a useful and rapid method that measures DNA damage in individual cells, using fluorescence microscopy and image analysis, and was originally described by Ostling and Johanson.[7)]

Olive and Durand reported that hypoxic cells could be detected using the comet assay, as these cells sustain approximately 3 times less DNA damage than aerobic cells on exposure to ionizing radiation.[8)] However, little is known about the relationship between the hypoxic fractions measured by radiobiological methods using rodent tumors and those detected by the comet assay.

Our previous study revealed that SCCVII tumors contain acutely hypoxic cells, whereas EMT6 tumors are chronically hypoxic.[9)] The clonogenic assay assumes that the vast majority of cells are either fully oxic or fully hypoxic. However, it is more likely that the cells exhibit a continuous distribution of oxygen tensions, and therefore exhibit a range of radiosensitivities,[3)] especially in chronically hypoxic EMT6 tumors.

We have modified an alkaline comet assay that determines single-strand breaks, using an original software program and a commercially available computer system, for identifying hypoxic fractions. We compared hypoxic fractions determined by the comet assay with those simultaneously measured by clonogenic assay using two rodent tumors, SCCVII/C3H and EMT6/KU/balb/c. A good correlation between these methods in different types of hypoxic cells would demonstrate that the comet assay is clinically available to measure the hypoxic fraction of solid tumors.

MATERIALS AND METHODS

Tumor cells and animals *In vitro*: SCCVII murine tumor (a squamous cell carcinoma of the C3H mouse) cells[10)] were cultured in Eagle's minimal essential medium (MEM, Nissui, Tokyo) supplemented with 12.5% fetal bovine serum (FBS, Equitech Bio, Inc., Ingram, TX) under humidified conditions at 37°C with 5% CO_2. The

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cell suspensions (20×10^4 cells/ml) were prepared by trypsinization from exponentially growing cultures immediately before use.

In vivo: SCCVII and EMT6/KU (a mammary sarcoma of the Balb/c mouse[10]) cell lines were used. They were maintained *in vitro* in Eagle's MEM supplemented with 12.5% FBS and collected from monolayer cultures. Approximately 1.0×10^5 cells were inoculated subcutaneously in both thighs of syngeneic 8-week-old female mice. After 12 or 21 days, experiments were performed with tumors of 8–12 mm or 15–20 mm in the longest diameter. Tumor weight measured after excision ranged from 0.21 to 1.1 g.

Asesssment of the relationship of the parameter for the comet assay with irradiation dose *in vitro* The cells (20×10^4 cells/ml) were suspended in ice-cold Hanks' solution and irradiated with 250 kV X-rays at 1.85 Gy/min. The irradiation dose was 0, 3, 6, 9 and 12 Gy. Immediately after the irradiation, the cells were analyzed by means of the comet assay.

Irradiation under hypoxic and aerobic conditions The cell suspensions in Hanks' solution, at a density of 20×10^4/ml, were poured into plugged glass flasks and flushed with a gas mixture of 95% N_2/5% CO_2 for 40 min at room temperature, as described.[11] Under this condition, the oxygen enhancement ratio was 2.9. After hypoxic conditions had been established, the flasks were put into a water bath (4°C) and irradiated. Simultaneously, the cell suspensions kept under oxic conditions for 40 min at room temperature were put into the water bath (4°C) and irradiated. Dose-response curves were determined for oxic and hypoxic conditions.

Detection of hypoxic fractions by the comet assay (*in vitro*) The cells irradiated (12 Gy) under oxic and hypoxic conditions were mixed at ratios of 1:0, 1:1, 2:1, 1:2 and 0:1. Then, the DNA damage was analyzed by means of the alkaline comet assay using the parameter of tail moment, and hypoxic fractions were calculated.

Detection of hypoxic fractions by the comet assay and clonogenic assay (*in vivo*) Groups of mice were killed by cervical dislocation 5 min before or immediately after irradiation, which was previously shown to be sufficient to cause cells to become radiobiologically hypoxic.[9] All the mice received whole-body irradiation without anesthesia or physical restraint at a dose of 12 Gy as described.[9] The apparatus and conditions for radiation were as for the *in vitro* experiments. The dose contribution was confirmed to be homogeneous in each tumor. Tumors were excised immediately after irradiation, minced with scissors on ice, and divided in two. One part was prepared as a single cell suspension by stirring for 20 min at 37°C in neutral protease solution containing 0.1% protease (type IX, Sigma Chemical Co., St. Louis, MO) and 0.2% $NaHCO_3$ in Eagle's MEM, and used for the *in vivo-in vitro* clonogenic assay. Cell survival was determined by the standard clonogenic assay.[10] The hypoxic fractions of each tumor were estimated as the proportions of the surviving fractions of the tumors in killed mice to those in living mice, instead of using the conventional paired survival curve method. The rest of the tumor was stirred for 20 min at 4°C in 0.02% ethylenediaminetetraacetic acid (EDTA)/phosphate-buffered saline (PBS) and used for the comet assay.

Alkaline comet assay The alkaline comet assay was performed essentially as described by Olive *et al*.[12] Five hundred microliters of the cell suspension from each group was mixed with 1.5 ml of 1% low gelling temperature agarose (type VII, Sigma Chemical Co.) in PBS maintained at 40°C and 20 μm microspheres (Coulter Co., Miami, FL), at a density of 1×10^4/ml. The microspheres were added to give the gel uniform thickness. Forty microliters of agarose mixed with each sample was immediately layered on a superfrosted glass microscope slide (Matunami Glass Ind., Ltd., Kishiwada) and covered with gelbond film (Pharmacia Biotech AB, Uppsala, Sweden). The slides were kept on ice for 1 min to allow the agarose to gel. Then the slides were removed from the gelbond film in PBS. The layer of agarose gel was attached to the gelbond film. Thereafter, the gelbond film was placed on 20 μl of 1% agarose with 20 μm microspheres on another glass microscope slide, taking care to avoid forming bubbles. Again, the slide was kept on ice, then removed. Next, double layers were formed on the gelbond film. These samples were lysed in a solution containing 0.03 mol/liter NaOH, 1 mol/liter NaCl and 0.5% sarcosyl, pH 12.5, for 50 min in the dark at room temperature and washed in a solution containing 0.03 mol/liter NaOH and 0.001 mol/liter EDTA for 1 h with three changes of the rinsing solution. The gelbond films were placed in a horizontal gel electrophoresis apparatus (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD). A fresh solution of 0.03 mol/liter NaOH and 0.002 mol/liter EDTA was added to the chamber such that the buffer level was about 5 mm above the agarose on the gelbond film, then electrophoresis was conducted at 0.5 V/cm for 20 min. After the electrophoresis, the samples were rinsed in PBS and stained for 20 min with 2.5 μg/ml propidium iodide in PBS. The stained gelbond films were rinsed in PBS and covered with glass on microscope slides. The slides were kept in a humidified, chilled and light-reduced environment and examined within 24 h.

Analysis of images The slides were viewed with a fluorescence microscope (BX-60, Olympus, Tokyo), under illumination with green light using a 530 nm reflector, with a 10× objective lens. Images were acquired with a charge-coupled device (CCD) camera (SenSys0400, Sekitecnotron, Tokyo) attached to the microscope. Images were randomly selected except for those with overlapping comets. Individual comets were transferred to a personal computer (Power Macintosh 8500/150, Apple Computer,

Tokyo) as a tag image file format (TIFF) image using conversion software (IPLab 3.1, Signal Analytics Co., Vienna, VA). The system was developed by us using the IDL software program (Research Systems, Inc., Boulder, CO). In this system, the background was subtracted using defined threshold values and the center of the tail (A) and head (B) were detected from the DNA intensity. The tail length and tail moment[12] (the product of the percentage of DNA in the tail multiplied by the distance between A and B) were calculated automatically.

RESULTS

Dose-response curve Fig. 1 shows the relationships between radiation dose and tail moment or tail length in the comet assay. The values of tail length and tail moment increased with dose from 0 up to 12 Gy.

Response to irradiation under oxic and hypoxic conditions Fig. 2 shows a microscopic image of comets irradiated at 12 Gy under oxic and hypoxic conditions. Fig. 3 shows the tail moment and tail length measured under oxic and hypoxic conditions as a function of radiation dose. The difference of tail moment between the oxic and hypoxic cells increased with the irradiation dose. At 12 Gy, the tail moment of hypoxic cells was 4.3 times less than that of oxic cells. However, there was no marked difference in tail length between hypoxic and oxic cells.

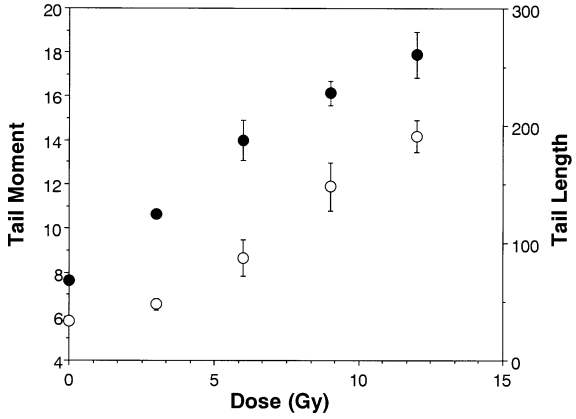

Fig. 1. Dose-response curve: the relationship between irradiation dose and the tail moment (open circles) or the tail length (closed circles). Data are the average (1 SE) values from four experiments.

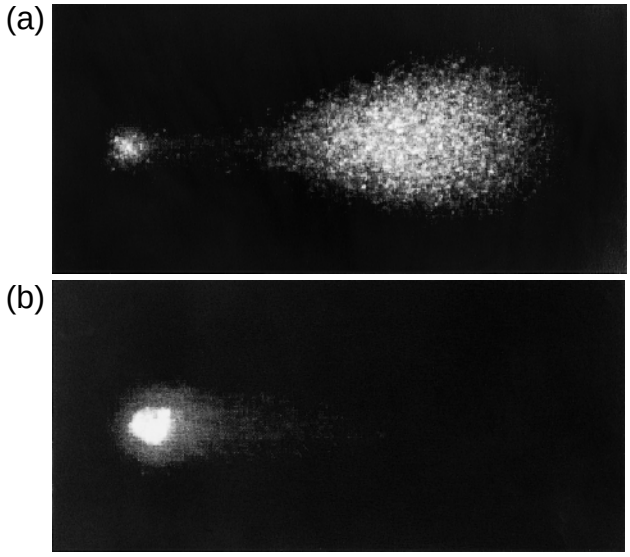

Fig. 2. The microscopic image of comets irradiated at 12 Gy under oxic (a) and hypoxic (b) conditions.

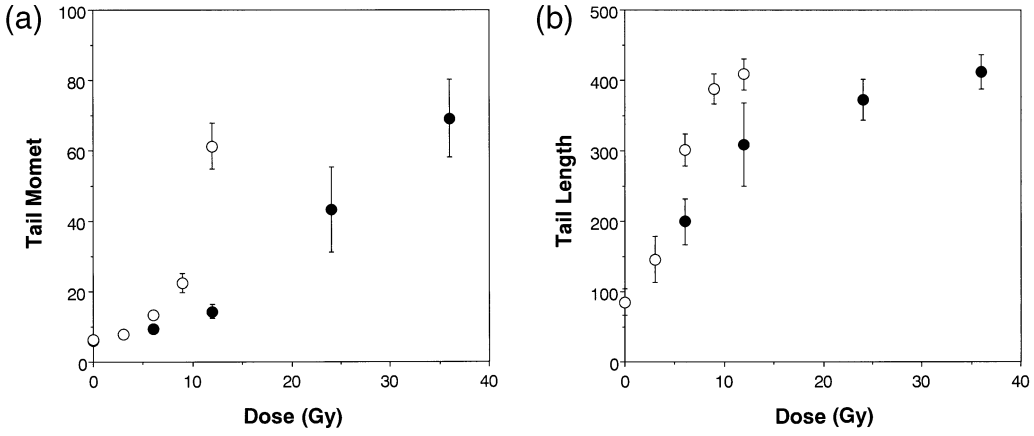

Fig. 3. Dose-response curve under oxic (open circles) and hypoxic (closed circles) conditions. (a) The relationship between irradiation dose and the tail moment. (b) The relationship between irradiation dose and the tail length. Data are the average (1 SE) values from four experiments.

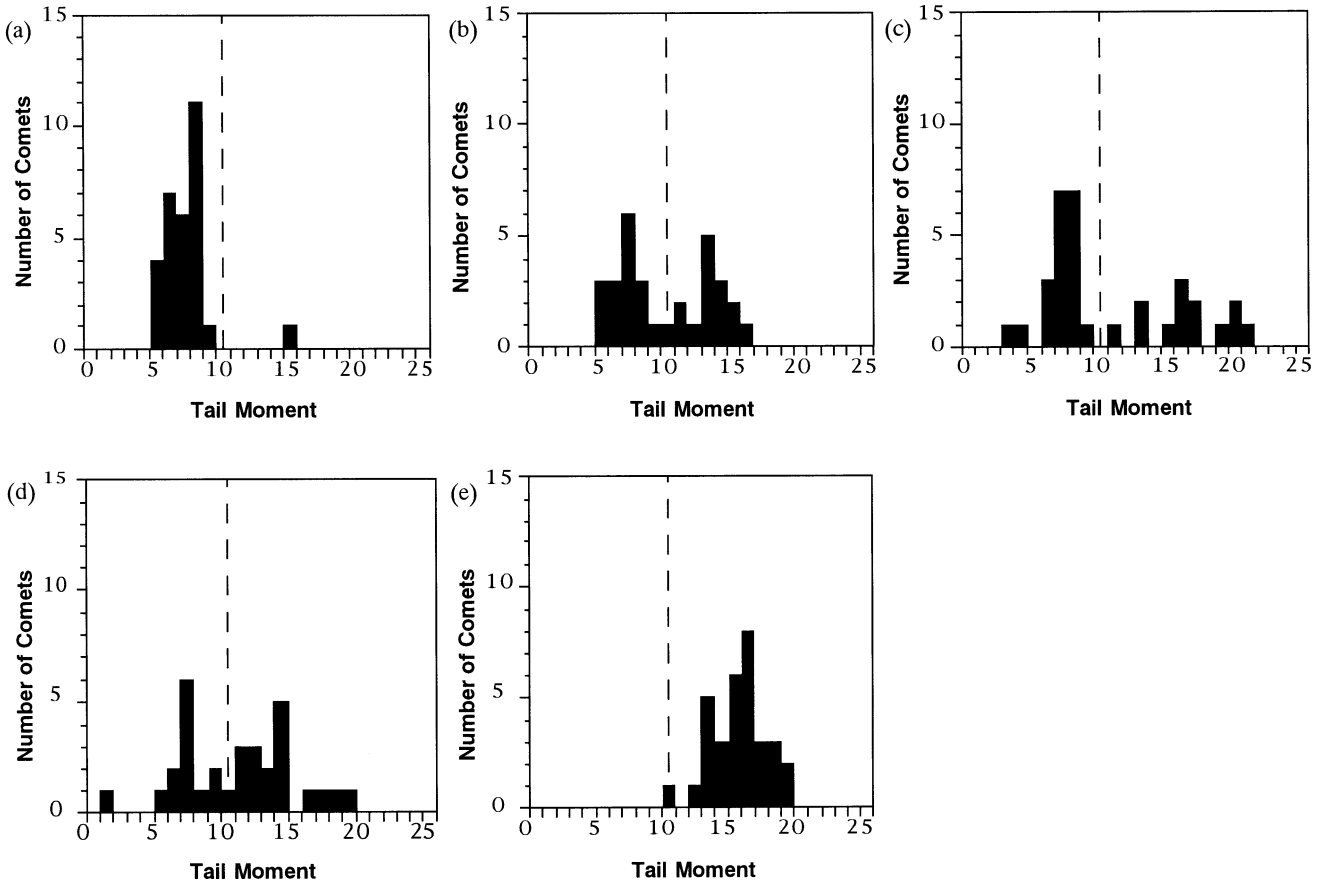

Fig. 4. Representative tail moment histograms analyzed by computer for irradiated (12 Gy) oxic and hypoxic mixed cell populations; (a) all hypoxic cells, (b) oxic cells:hypoxic cells=1:1, (c) oxic cells:hypoxic cells=1:2, (d) oxic cells:hypoxic cells=2:1, (e) all oxic cells. A vertical line was drawn arbitrarily to allow easy identification of the hypoxic fraction.

Detection of hypoxic fractions *in vitro* We evaluated the tail moment at 12 Gy to detect the hypoxic fraction by means of the comet assay *in vitro*. Fig. 4 shows representative tail moment histograms for oxic and hypoxic cells mixed at various ratios (0:1, 1:1, 2:1, 1:2, 1:0) and exposed to 12 Gy. For each sample, at least 50 cells were analyzed. In almost all hypoxic cells, the values of tail moment were smaller than 10 (Fig. 3a). In contrast, in hypoxic cells, they were larger than 10. In the mixed populations, there were two peaks. We judged those cells with a tail moment smaller than 10 to be hypoxic and calculated the hypoxic cell fractions from these histograms. The values of tail moment were different in each experiment, but the tendency was similar. There was a good correlation between the hypoxic fractions measured using the comet assay and known percentages (Fig. 5).

Detection of hypoxic fractions *in vivo* Using the comet assay, we measured the hypoxic fractions of SCCVII tumors grown subcutaneously in the thigh of C3H/He

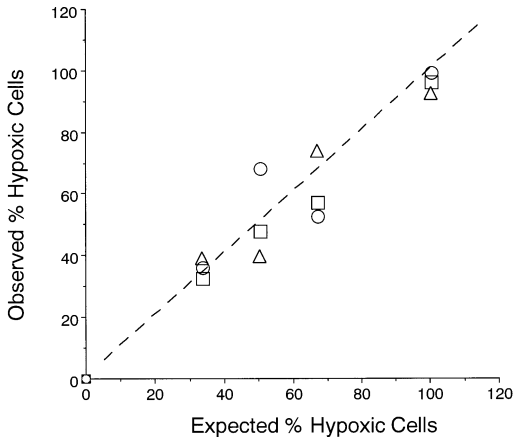

Fig. 5. Detection of hypoxic cells by the comet assay in known mixtures of aerobic and hypoxic SCCVII tumor cells. The results of three experiments are shown (open squares, Ex.1; open triangles, Ex.2; open circles, Ex.3).

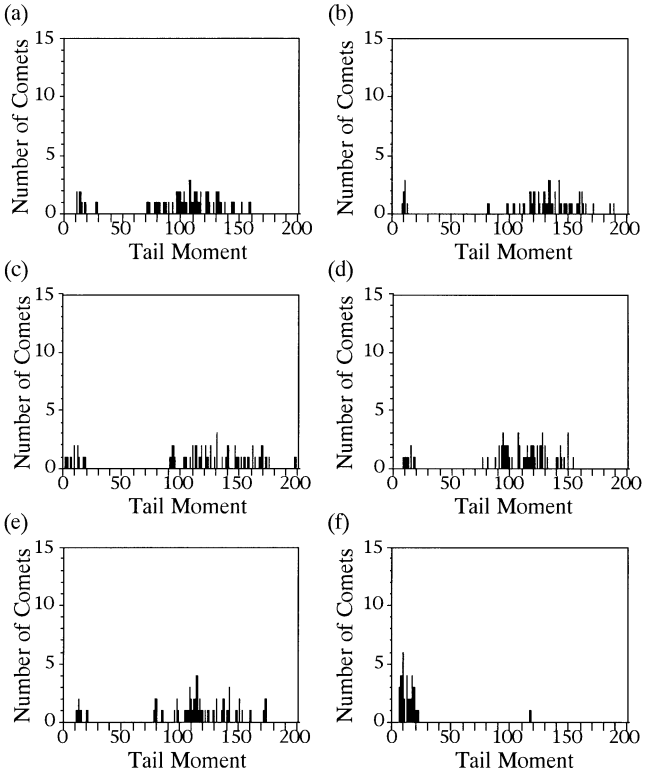

Fig. 6. Representative distribution of tail moment values among tumor cells (SCCVII) grown subcutaneously in the thigh of C3H/He mice exposed to 12 Gy immediately before (a–e) or 5 min after (f) being killed by cervical dislocation. Each graph was made from the values for one mouse.

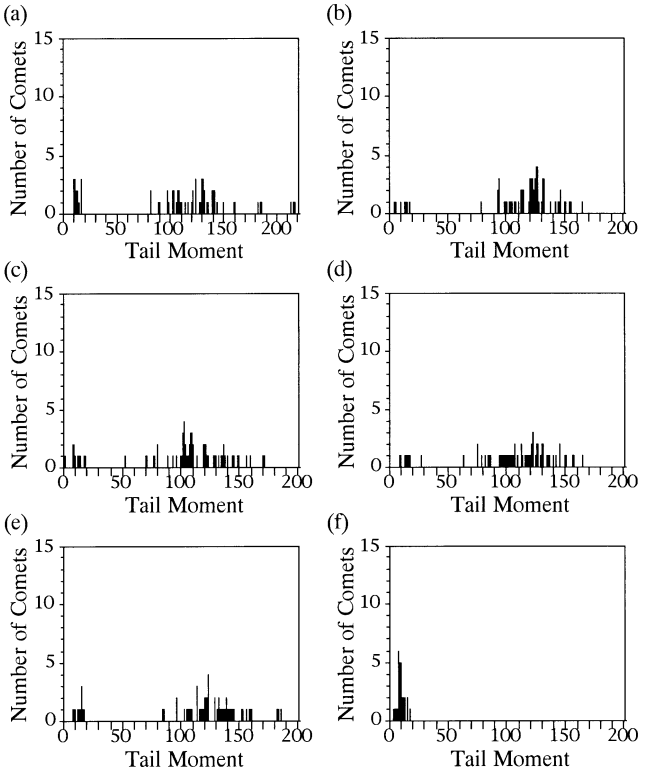

Fig. 7. Representative distribution of tail moment values among the tumor cells (EMT6/KU) grown subcutaneously in the thigh of Balb/c mice exposed to 12 Gy immediately before (a–e) or 5 min after (f) being killed by cervical dislocation.

mice and EMT6 tumors grown in Balb/c mice. Simultaneously each tumor was examined for a hypoxic fraction using the clonogenic assay and the results were compared with those obtained using the comet assay. Figs. 6 and 7 show the distribution of tail moment values among the tumor cells obtained from the mice exposed to 12 Gy immediately before or 5 min after being sacrificed by cervical dislocation.

The DNA damage was extensive and the values of tail moment were larger than those for the *in vitro* experiments. However, in each tumor, there were two groups of cells; those in which the tail moment was larger than 50 and those in which it was smaller than 50. The tail moment values of the mice killed 5 min before irradiation were smaller than 50 (Figs. 6 and 7). The former cells were regarded as oxic and the latter as hypoxic, and the hypoxic fraction was calculated. Fig. 8 shows the relation between the hypoxic fractions determined by the comet assay and those measured by the clonogenic assay. There was a significant correlation between them in SCCVII (r=0.85, P<0.01) and EMT6 (r=0.75, P<0.01).

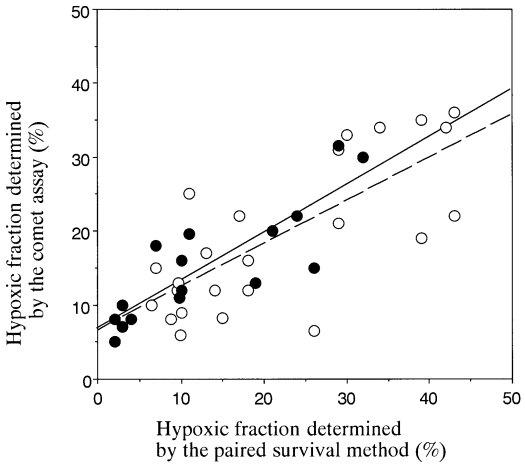

Fig. 8. The relationship between the hypoxic fraction determined by the comet assay and the paired survival method in SCCVII tumors (closed circles and solid line) and EMT6/KU tumors (open circles and dashed line). There were significant correlations (SCCVII: r=0.85, P<0.01; EMT6: r=0.75, P<0.01).

DISCUSSION

The alkaline comet assay has been introduced as a method for measuring DNA damage (single strand breaks) in individual cells, rapidly and with a high sensitivity. In this study, we developed an image analysis system and modified the comet assay for the purpose of detecting hypoxic fractions of solid tumors.

The method to make gel layers with microspheres was originally developed by Furusawa *et al.* (personal communication). The technique prevents the cells from flowing out of the gel or being damaged by surface tension. Moreover, the thickness of the gel is uniform and the comet image on fluorescence microscopy is very clear. Our findings on the relationship between the tail moment (or tail length) and radiation dose were similar to those previously reported[12] (Fig. 1), although the value of the parameter and the rate of increase were different, which may be due to electrophoresis or lysing conditions (buffer, voltage and temperature).

Ionizing radiation produces approximately 3 times fewer DNA strand breaks in hypoxic than in oxic cells,[13] which is the principal behind the detection of hypoxic cells by the comet assay. However, in the comet assay, the value of the parameter of oxic cells is not necessarily three times larger than that of hypoxic cells. In our *in vitro* experiments, using SCCVII tumors, the difference of tail moment between oxic and hypoxic cells became larger with the radiation dose. As the cells became intensely damaged and the tail deformed with a spindle shape (Fig. 2a), the value of tail moment increased markedly, with little change in the tail length.

One reason why the difference of tail moment was greater at a high irradiation dose may be that, in less damaged hypoxic cells, rejoining of single-strand breaks may occur more rapidly than in heavily damaged oxic cells, although this is rather unlikely, given the effort made to prevent repair.

Cook *et al.* observed that, when DNA was broken by irradiation, the supercoiling relaxed and loops formed a 'halo' around the nucleoid core.[14] Collins *et al.* assumed that the number of loops in the tail indicates the number of DNA breaks.[15] Alkaline electrophoresis is followed by neutralization, so they suggested that, while the DNA of the head is able to reanneal, the strands of relaxed and broken tail loops separate in alkali and so reassociation is unlikely. This appears to be supported by our finding that the tail density rather than tail length increased markedly with greater damage. The difference in the tail length between oxic and hypoxic cells was too small to enable detection of hypoxic fractions.

These factors should be strongly influenced by the electrophoresis or lysing conditions, concentration of agarose and temperature. Therefore, the parameters used in the present study are relative, not absolute, values. At low dose irradiation, the difference in the tail moment between oxic and hypoxic cells was very small, making differentiation more difficult.

Using cultured tumor cells (SCCVII), we calculated the artificial hypoxic fractions by measuring the tail moment of oxic and hypoxic cells. There was a good correlation between hypoxic fractions measured using the comet assay and known percentages. Our alkaline comet assay could detect hypoxic cells with good sensitivity *in vitro*.

In vivo, sampling methods are critical to the measurement of hypoxic fractions. In our experiments, excised tumors were minced, enabling the entire tumor cell population to be sampled. The samples to be used for clonogenic assay were stirred in 37°C neutral protease solution to release the cells. However, with this method, we could not detect hypoxic cells by the comet assay, because the tail moment of almost all cells was as small as that of non-irradiated cells (data not shown). While the cells are being stirred at 37°C, rejoining of single-strand breaks might occur. Consequently, we used 0°C EDTA/PBS for the comet assay. The value of tail moment was much greater in these experiments than in the former more basic experiments *in vitro*. This tumor suspension procedure may further damage the DNA of oxic cells.

Olive *et al.* used fine-needle aspirates for the comet assay, because only a few thousand cells are required. It is suggested that this method is superior to excision biopsy, especially clinically, in terms of speed, accessibility, convenience, and the fact that disaggregation of the specimen is not required.[16] However, they reported that the percentage of circulating white blood cells increased in fine needle aspirates especially when multiple samples were removed from a single tumor and there was significant bleeding. Hu *et al.* suggested that the force involved in the aspiration may increase the DNA damage.[17] It is necessary to develop better sampling techniques to improve the accuracy of the comet assay.

We compared hypoxic fractions determined by the comet assay with those simultaneously measured using a radiobiological method, *in vivo*. A good correlation was observed in two rodent tumors, SCCVII and EMT6, which have different types of hypoxia. According to our previous study, EMT6 tumor had a tendency to become chronically hypoxic, whereas SCCVII tumors contained acutely hypoxic cells.[9] In EMT6 tumor, significant proportions of the cells may be moderately hypoxic and therefore have intermediate radiosensitivities. Thus some moderately damaged cells may be detected by the comet assay. However, we observed only two patterns of cell damage: slight and marked. The hypoxic fractions calculated by the comet assay well reflected those detected radiobiologically in both tumors.

Recently, a new electrode system has become available, having undergone extensive clinical testing.[18] However, with this method pO_2 measurements were not consistently related with radiobiological hypoxic fractions. Fenton *et al.* suggested that, even though a large fraction of a tumor is anoxic, the radiobiological hypoxic fraction may still be small if a substantial number of nonclonogenic anoxic cells are present.[19] Sasai and Brown suggested that cell survival depended on the oxygen tension in individual cells, whereas polarographic measurements of oxygen tension by electrodes yields an average measurement over many cells.[20]

In conclusion, we compared hypoxic fractions determined by the comet assay, which detects the DNA damage in individual cells, with those measured by clonogenic assay, the endpoint of which is clonogenic potential. Results obtained with these two methods showed a good correlation in two rodent tumors with different types of hypoxia. Thus, this comet assay may be useful for estimating hypoxic fractions of solid tumors. Improving the system and sampling techniques for the comet assay should make it more applicable to the estimation of radiobiological hypoxic fractions of solid tumors. We are now planning to measure hypoxic fractions of human tumors.

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