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Radiobiological characteristics of solid tumours depending on the p53 status of the tumour cells, with emphasis on the response of intratumour quiescent cells

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Abstract

Human head and neck squamous cell carcinoma cells transfected with mutant *TP53* (SAS/m*TP53*) or with a *neo* vector as a control (SAS/*neo*) were inoculated subcutaneously (s.c.) into both hind legs of Balb/cA nude mice. Mice bearing tumours received 5-bromo-2′-deoxyuridine (BrdU) continuously to label all proliferating (P) cells in the tumours. The mice then received γ-ray irradiation. Another group of mice received a series of test doses of γ-rays while alive or after tumour clamping to obtain hypoxic fractions (HFs) in the tumours. Right after irradiation, the tumour cells were isolated and incubated with a cytokinesis blocker. The micronucleus (MN) frequency in the cells without BrdU labelling (=quiescent (Q) cells) was determined using immunofluorescence staining for BrdU. Meanwhile, 6 h after irradiation, tumour cell suspensions obtained in the same manner were used for determining the frequency of apoptosis in the Q cells. The MN frequency and apoptosis frequency in total (P+Q) tumour cells were determined from the tumours that were not pretreated with BrdU. In total cell populations, SAS/m*TP53* cells were more radioresistant than SAS/*neo* cells in clonogenic survival. Q tumour cells exhibited a significantly lower apoptosis and MN frequency, probably due to their much larger HF, than total cells. In both total and Q cell fractions, SAS/m*TP53* cells were less susceptible to apoptosis and more susceptible to micronucleation than SAS/*neo* cells. Obviously, *TP53* status had the potential to influence the radiosensitivity of not only the total cells, but also the Q cells. However, irrespective of the *TP53* status, significant differences in radiosensitivity between total and Q tumour cells were consistently observed. From the viewpoint of tumour control as a whole, including intratumour Q tumour cell control, a treatment modality for enhancing the Q cell response has to be considered.

Keywords: Quiescent cell; p53 status; Apoptosis; Micronucleus; Hypoxic fraction; γ-Rays; Radiosensitivity; BrdU

1. Introduction

Recently, it has been shown that the *TP53* tumour suppressor gene serves a critical role in maintaining genomic stability during a cell cycle checkpoint in the G1 and G2/M transitions [1–3], as an effector of DNA repair [4,5] and apoptosis [6,7]. Wild-type p53 is needed to activate apoptosis in sensitive cells in response to DNA damage [8]. These actions of p53 are potentially critical in determining the effectiveness of ionising radiation and/or chemotherapeutic agents. Actually, mutations in the *TP53* tumour suppressor gene have been shown to have an impact on the clinical course of several human cancers: cancer patients with *TP53* mutations in their tumours often have a worse prognosis than those with wild-type *TP53* [9]. Thus, the genetic and functional status of *TP53* is thought to be an important factor in guiding therapeutic strategies for cancer patients.

Many of the cells in solid tumours are non-proliferating (quiescent (Q)). Over the past 25 years, the characteristics of Q cells have been examined extensively, but there is still much to be learned [10,11]. To improve cancer treatments, the response of Q cells in solid tumours to various anticancer treatments should be determined, since many tumour cells are quiescent *in*

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situ, but are still clonogenic [10,11]. Until recently, a simple assay for assessing the response of intratumour Q cells was not available. In analysing the response of Q cells in solid tumours, we have developed a method utilising both the micronucleus (MN) assay and the identification of proliferating (P) cells by 5-bromo-2′-deoxyuridine (BrdU) and anti-BrdU monoclonal antibody [12]. Micronuclei have been reported to appear in dividing cells with chromosomal aberrations after irradiation. The frequency of their appearance was reported to correlate with the dose of radiation and extent of cell killing [13,14]. However, subsequent research has shown that some cells die through apoptosis after irradiation and that this may be related to the tumour sensitivity to irradiation [15]. Very recently, our method for detecting the Q cell response to irradiation using P cell labelling with BrdU and the MN frequency assay was also shown to be applicable to the detection of apoptosis [16].

Therefore, we examined the relationship between the radiobiological characteristics of tumour cells, that is, total (P + Q) cells and Q cells, in solid tumours and the *TP53* status of the cells constituting the solid tumours, using tumour cell lines with identical genetic backgrounds except for their *TP53* status.

2. Materials and methods

2.1. Cells, tumours and mice

The human head and neck squamous cell carcinoma cell line SAS (provided by JCRB, Tokyo Japan) was cultured at 37 °C in Dulbecco's Modified Eagle medium containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) and 12.5% (v/v) fetal bovine serum (DMEM) in a conventional humified 5% (v/v) CO_2 incubator. SAS cells show the phenotype of wild-type *TP53* in radiation- and heat-induced signal transduction [17,18], although they have a point mutation at codon 336 of exon 10 in *TP53* [19]. Plasmids pC53-248, which contains a m*TP53* gene (codon 248, from Arg to Trp) producing a dominant-negative mp53 protein, and pCMV-*Neo*-Bam, which contains a neo-resistant marker, were provided by B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD, USA). These plasmids were linearised with *HindIII*. Confluent SAS cells, approximately 2×10^6 cells in a 75-cm^2 flask were trypsinised, and the resulting cell suspension in phosphate-buffered saline (PBS) (1 ml) was transferred into an electroporation chamber. Cells were supplemented with linearised DNA (10 μg/10 μl of pC53-248 or pCMV-*Neo*-Bam), and electroporated three times at 600 V. After standing for 30 min at room temperature, cells were plated onto dishes 10 cm in diameter in DMEM and incubated at 37 °C. Forty-eight hours later, cells were treated with G418 (geneticin, 200 μg/ml, Sigma Chemical Co. St. Louis, MO, USA), an agent for the selection of the transfected clones, and then incubated at 37 °C for 14 days to allow colony formation. Colonies resistant to G418 were isolated with cloning cylinders. Through these manipulations, two stable transfectants SAS/m*TP53* and SAS/*neo* were established. SAS/*neo* cells have a functionally wild-type p53 protein, and SAS/mTP53 cells express a dominant-negative p53 protein. The procedure used for transfection is described in detail elsewhere in Ref. [20].

Cells were collected from exponentially growing cultures, and approximately 5.0×10^5 cells were inoculated subcutaneously (s.c.) into both hind legs of 6- to 7-week-old syngeneic female Balb/cA nude mice. Three weeks after inoculation, a tumour with a diameter of approximately 7 mm could be observed at each implanted site, whichever stable transfectant was used.

2.2. Determining the timing for apoptosis detection

γ-Rays at a dose of 20 Gy from a cobalt-60 γ-ray irradiator at a dose rate of 5.97 Gy/min were given to the tumour-bearing Balb/cA nude mice 3 weeks after tumour cell inoculation. At various time points after irradiation, mice were sacrificed, and tumours were excised. Apoptosis induced in the tumours was detected by the following two methods.

2.2.1. Deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) method (ApopTag, Oncor)

Tumour samples were fixed routinely in 10% (v/v) formalin overnight at room temperature, embedded in paraffin, and cut into 4-μm-thick sections. The sections were deparaffinised in xylene, dehydrated through graded alcohol, and washed in 0.01 M PBS. The sections were incubated with 20 mg/ml proteinase K for 40 min. They were incubated with equilibration buffer followed by terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37 °C for 1 h, and then a stop/wash buffer was applied for 30 min at 37 °C. They were incubated with anti-digoxigenin-peroxidase for 30 min at room temperature. The sections were counterstained with haematoxylin.

2.2.2. Fluorescence staining method

Excised tumours were minced, and trypsinised at 37 °C for 15 min, using 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA). Tumour cell suspensions were fixed with 70% (v/v) ethanol overnight at 4 °C. After centrifugation, the cell pellet was resuspended in 0.4 ml of cold modified Carnoy's fixative (three volumes of ethanol and one volume of acetic acid). The suspension was then placed on a glass microscope slide using a dropper and the sample was dried at room temperature. Cells on the slides were treated with 30 μl of

propidium iodide (PI, 1–5 μg/ml in PBS) and monitored under a fluorescence microscope.

2.2.3. Determination of apoptosis

With the TUNEL method, the apoptosis frequency was expressed as the number of positively stained tumour cells and bodies with the morphological characteristics of apoptosis among 3000 tumour cells. With the fluorescence method, tumour cells and bodies with apoptotic characteristics were enumerated among at least 600 tumour cells. Standard criteria for the morphological characteristics of apoptosis were chromatin condensation, nucleolar disintegration and the formation of crescent caps of condensed chromatin at the nuclear periphery [21,22]. Single, relatively large ($\geqslant$4 μm in diameter) and roundish nuclear residues existing in extra- or intratumoral cells with intensive staining were identified as apoptotic bodies.

2.3. Labelling with BrdU

Two weeks after tumour cell inoculation, mini-osmotic pumps (Alzet model 2001, Palo Alto, CA, USA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted s.c. to label all of the P cells for 7 days. Administration of BrdU did not change the tumour growth rate. The tumours were approximately 7 mm in diameter on treatment. The labelling index (LI) after continuous labelling with BrdU was 48.4% (41.7–55.1%) (mean (95% Confidence Limit (CL))) and 43.2% (37.0–49.4%) for SAS/*neo* and SAS/m*TP53* tumour cells, respectively, and reached a plateau level at these stages. Therefore, in this study, we regarded tumour cells not incorporating BrdU after continuous labelling as Q cells.

2.4. Treatment

2.4.1. Irradiation with γ-rays

After labelling with BrdU, the tumour-bearing mice received whole-body irradiation at a dose of 4–25 Gy using a cobalt-60 γ-ray irradiator at a dose rate of 5.97 Gy/min.

2.4.2. γ-Ray test irradiation for determining the hypoxic fraction (HF)

Another group of tumour-bearing mice received a series of test radiation doses of 18–22 Gy from the cobalt-60 γ-ray irradiator. Tumours in one group of mice were made hypoxic by clamping the proximal end 15 min before the irradiation, as previously reported in Ref. [23]. This clamping method did not influence cell survival or the levels of apoptosis and micronuclei. Immediately after irradiation, the clamp was released. Tumours in another group received irradiation without clamping.

Each group for irradiation and γ-ray test irradiation included mice pretreated with and without BrdU.

2.5. Immunofluorescence staining of BrdU-labelled cells and observation of apoptosis and micronucleation

Based on the time-course of the change in apoptosis frequency, 6 h after irradiation for the apoptosis assay, and right after irradiation for the micronucleus (MN) assay, tumours were excised from mice given BrdU, minced and trypsinised. For the apoptosis assay, tumour cell suspensions thus obtained were fixed with 70% (v/v) ethanol overnight at 4 °C. For the MN assay, tumour cell suspensions were incubated for 48 h in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis, while allowing nuclear division, and the cultures were then trypsinised and cell suspensions were fixed. For both assays, after centrifugation of fixed cell suspensions, the cell pellet was resuspended with cold Carnoy's fixative. The suspension was then placed on a glass microscope slide and the sample was dried at room temperature. The slides were treated with 2 N hydrochloric acid for 45 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in borax-borate buffer (pH 8.5) to neutralise the acid. BrdU-labelled tumour cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody (2% (v/v), Becton Dickinson, San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (2% (v/v), Sigma, St. Louis, MO, USA). To observe double staining of tumour cells with green indicating FITC and red indicating PI, cells on the slides were treated with PI and monitored under a fluorescence microscope.

The frequency of apoptosis in the BrdU-unlabelled cells (=Q cells) was determined by counting apoptotic cells among tumour cells that showed only red fluorescence. The frequency was defined as the ratio of the number of apoptotic cells to the total number of tumour cells observed. The MN frequency in the BrdU-unlabelled cells was examined by counting the micronuclei in the binuclear cells that showed only red fluorescence. The MN frequency was defined as the ratio of the number of micronuclei in the binuclear cells to the total number of binuclear cells observed [16].

The ratios obtained in tumours not pretreated with BrdU indicated the apoptosis frequency and the MN frequency at all phases in the total (P+Q) tumour cell populations. More than 300 tumour cells and binuclear cells were counted to determine the apoptosis frequency and the MN frequency, respectively.

2.6. Measurement of HF in tumours from mice in the γ-ray test irradiation group

In the γ-ray test irradiation group, the cell survival assay was also performed in mice given no BrdU using an *in vivo–in vitro* assay method. Tumours were disaggre-

gated by stirring for 20 min at 37 °C in PBS containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. The cell yield was 1.5×10^7/g ($1.2–1.8\times10^7$/g) and 3.4×10^6/g ($2.6–4.2\times10^6$/g) for SAS/*neo* and SAS/m*TP53* tumours, respectively.

The values of the apoptosis frequency and the MN frequency in BrdU-unlabelled tumour cells were extrapolated to the surviving fraction (SF), using the regression line for the relationships between the normalised apoptosis frequency and the SF and between the normalised MN frequency and the SF determined for total cells in tumours from mice that were not pretreated with BrdU, respectively. The normalised apoptosis and MN frequencies were the apoptosis and MN frequency in tumours that received γ-ray irradiation minus the apoptosis and the MN frequency in tumours that did not receive γ-ray irradiation, respectively. Therefore, the cell survival curves of BrdU-unlabelled cells (=Q cells) were determined under both aerobic and hypoxic conditions.

To determine the HF of the tumours from mice in the γ-ray test irradiation group, the paired survival curve method was employed [24]. Hypoxia was induced by clamping the proximal end of tumours 15 min before irradiation. After deviations from linearity and parallelism had been tested for the dose-survival curves under both aerobic and hypoxic conditions, the best linear parallel lines were fitted to the two survival curves by least squares regression, and the HFs were determined from the vertical displacement of the parallel lines.

To confirm the stability of transfectants SAS/*neo* and SAS/m*TP53*, part of tumour cell suspensions obtained after γ-ray irradiation to solid tumours and tumour cells from part of the colonies grown through the *in vivo–in vitro* assay method were subjected to Western blotting analysis for p53 and Bax proteins as described in Ota and colleagues [17]. Not only the p53 protein level, but also its function can be detected because the *bax* gene is a p53-target gene. As a result, it was certified that the p53 status of each transfectant was not changed by these experimental procedures. Three mice were used to assess each set of conditions and each experiment was repeated three times. To examine the differences between pairs of values, Student's *t*-test was used when variances of the two groups could be assumed to be equal, otherwise the Welch *t*-test was used. *P* values were from two-sided tests.

3. Results

Table 1 shows the plating efficiencies for the total tumour cells and the apoptosis frequencies and the MN frequencies for the total and Q tumour cells, when irradiation was not administered. Under all conditions, SAS/*neo* had a significantly higher plating efficiency and apoptosis frequency than SAS/m*TP53* ($P<0.05$). In contrast, in MN frequency, SAS/m*TP53* had significantly higher values than SAS/*neo* ($P<0.05$). In both cell lines, Q cells showed significantly higher apoptosis

Table 1
Plating efficiencies, apoptosis frequencies and micronucleus frequencies at 0 Gy

	Total cells	Quiescent cells
Plating efficiency (%)		
Aerobic condition		
SAS/*neo*	45.4 (36.3–54.5)[a]	–
SAS/m*TP53*	23.5 (17.4–29.6)	–
Hypoxic condition (clamping)		
SAS/*neo*	44.3 (35.4–53.2)	–
SAS/m*TP53*	22.5 (16.4–28.6)	–
Apoptosis frequency		
Aerobic condition		
SAS/*neo*	0.083 (0.076–0.090)	0.106 (0.091–0.121)
SAS/m*TP53*	0.009 (0.007–0.011)	0.033 (0.029–0.037)
Hypoxic condition (clamping)		
SAS/*neo*	0.091 (0.083–0.099)	0.116 (0.100–0.132)
SAS/m*TP53*	0.011 (0.008–0.014)	0.039 (0.034–0.044)
Micronucleus frequency		
Aerobic condition		
SAS/*neo*	0.038 (0.032–0.044)	0.056 (0.049–0.063)
SAS/m*TP53*	0.072 (0.064–0.080)	0.111 (0.101–0.121)
Hypoxic condition (clamping)		
SAS/*neo*	0.041 (0.034–0.048)	0.068 (0.060–0.076)
SAS/m*TP53*	0.091 (0.082–0.100)	0.133 (0.122–0.144)

[a] Numbers in parentheses are 95% Confidence Limits (CL), determined using mean values, standard deviations, and the numbers of observations on which the means and standard deviations were based.

and MN frequencies than total cells under all conditions ($P<0.05$). There was a tendency for the hypoxic conditions to produce lower plating efficiencies and higher apoptosis and MN frequencies than the aerobic conditions in both cell lines.

Fig. 1 shows the time course of the change in the apoptosis frequency determined by the TUNEL method and the fluorescence staining method for each cell line following γ-ray irradiation at a dose of 20 Gy. Whichever method was used, the apoptosis frequency showed a similar profile and a maximum value 6 h after irradiation in each cell line. On the whole, the apoptosis frequency for SAS/m*TP53* cells was very small, much smaller than that for SAS/*neo* cells ($P<0.05$).

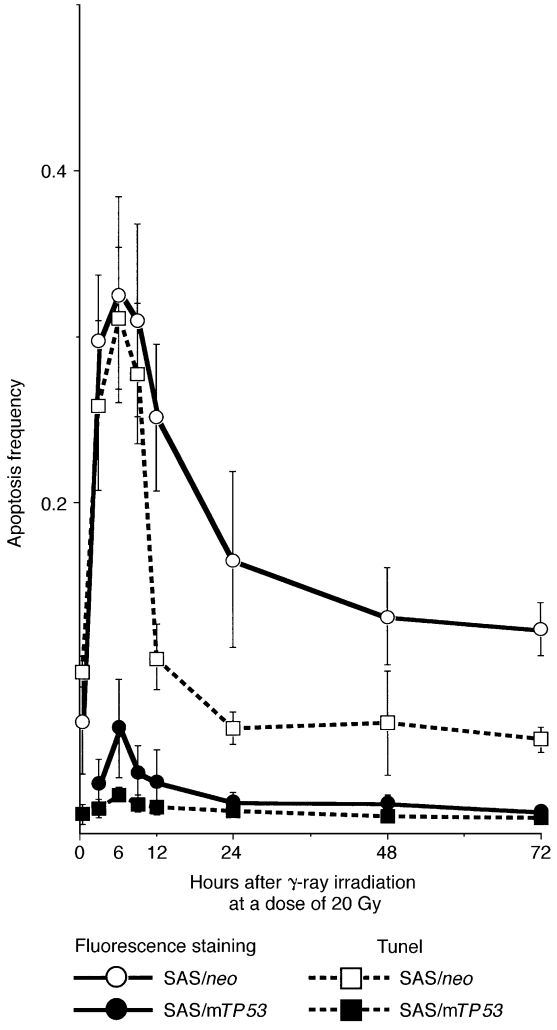

Fig. 1. Effect of time after γ-ray irradiation at a dose of 20 Gy on apoptosis frequency. The time course changes in the value of the apoptosis frequency for SAS cells transfected with *neo* vector (SAS/*neo*) (open symbols) and SAS cells transfected with mutant p53 (SAS/m*TP53*) (closed symbols) tumour cell lines following irradiation determined by the fluorescence staining method (circles) and the TUNEL method (squares). Bars represent 95% Confidence Limits (CL).

Fig. 2 shows the cell survival curves of total tumour cell populations after γ-ray irradiation for each cell line. The SAS/m*TP53* cells were approximately 1.5 times more resistant to γ-rays than the SAS/*neo* cells at the D10 dose. At doses over 18 Gy, the slopes of both curves started to plateau out and were almost parallel.

Figs. 3 and 4 show the normalised apoptosis and MN frequencies for both cell lines following γ-ray irradiation, respectively. For baseline correction, we used the normalised frequencies to exclude the effect of spontaneously induced frequencies in non-irradiated control tumours. The normalised frequency was the frequency in the irradiated tumours minus that in the non-irradiated tumours. In both cell lines, total tumour cells had significantly higher normalised frequencies than Q tumour cells ($P<0.05$). In both the total and Q cell fractions, SAS/*neo* showed significantly higher apoptosis frequencies than SAS/m*TP53* ($P<0.05$). However, SAS/m*TP53* showed significantly higher MN frequencies

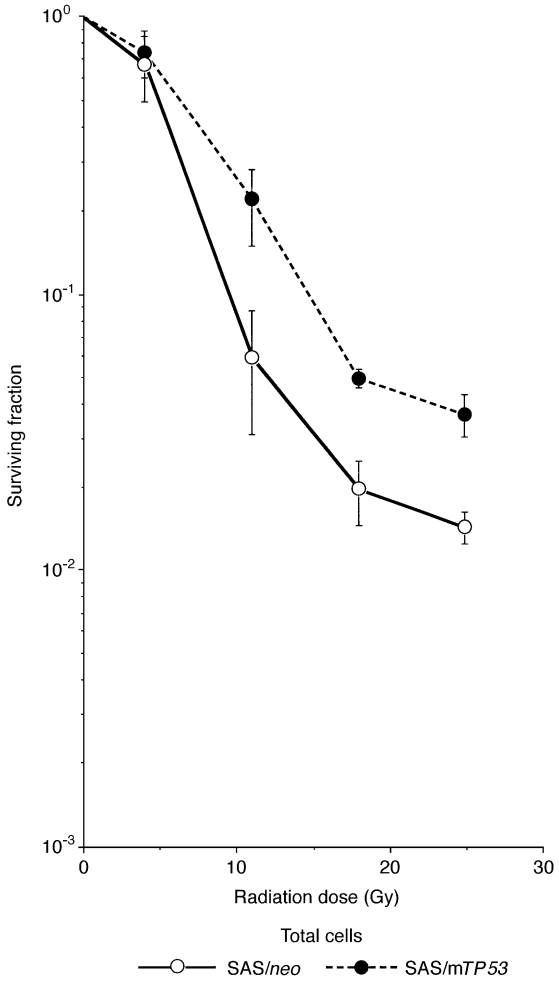

Fig. 2. γ-Ray radiation dose-response curves for total tumour cells of SAS cells transfected with *neo* vector (SAS/*neo*) (open circles) and SAS cells transfected with mutant p53 (SAS/m*TP53*) (closed circles) tumours. Tumour-bearing mice were irradiated while alive. Bars represent 95% Confidence Limits (CL).

for total cells ($P<0.05$), and higher MN frequencies for Q cells, although not significantly, than SAS/*neo*. We calculated the dose-modifying factors (DMFs) for Q cells and used them to compare the doses of γ-ray radiation necessary to obtain various normalised frequencies in Q cells with the doses required in total tumour cells, using the data shown in Figs. 3 and 4 (Table 2). Whichever normalised frequency was used as an end-point, the values of the DMF for Q cells were significantly larger than 1.00 ($P<0.05$) and showed a similar tendency in each tumour cell line.

For tumour-bearing mice in the γ-ray test irradiation group, the correlation between the normalized apoptosis or MN frequency and the SF of total tumour cells for each tumour was examined. In each tumour cell line, all regression lines had significant correlations ($P<0.001$) (Fig. 5). The SFs of the Q cells were obtained using the normalised apoptosis or MN frequencies of Q cells and the regression lines for total tumour cells. Fig. 6(a) and (b) show the survival curves for SAS/*neo* and SAS/m*TP53*, respectively. On the whole, the SFs of the Q cells were significantly larger than those of the total cells ($P<0.05$), especially in normally aerated tumours. Furthermore, the slopes of the survival curves for total cells were slightly steeper than those for the Q cells in both tumours. The HFs of total and Q tumour cell populations in normally aerated tumours were calculated from the best paired survival curves obtained by comparison between normally aerated tumours and hypoxic tumours using the data in Fig. 6.

Based on the directly determined SFs of total tumour cells, the HF in total cells was 7.9% (4.9–10.9%) and 14.1% (10.9–17.3%) for SAS/*neo* and SAS/m*TP53* tumours, respectively. Based on the SFs of the Q cells extrapolated from the normalised apoptosis frequency of the Q cells and the regression lines for the relationship between the normalised apoptosis frequency and

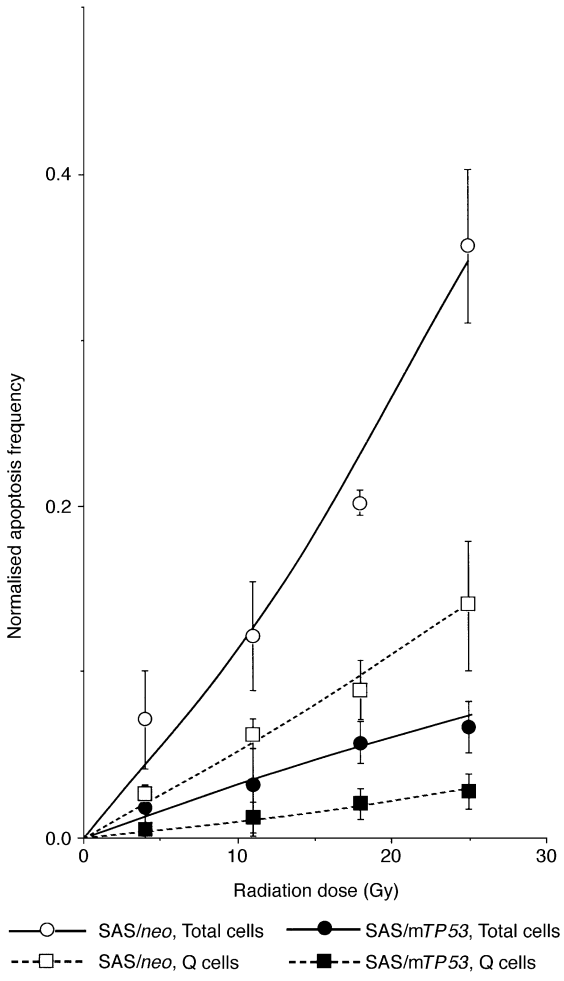

Fig. 3. Normalised apoptosis frequencies for total (circles) and quiescent (Q) (squares) cells of SAS cells transfected with *neo* vector (SAS/*neo*) (open symbols) or SAS cells transfected with mutant p53 (SAS/m*TP53*) (solid symbols) tumour cell lines following γ-ray irradiation. Bars represent 95%. Confidence Limits (CL).

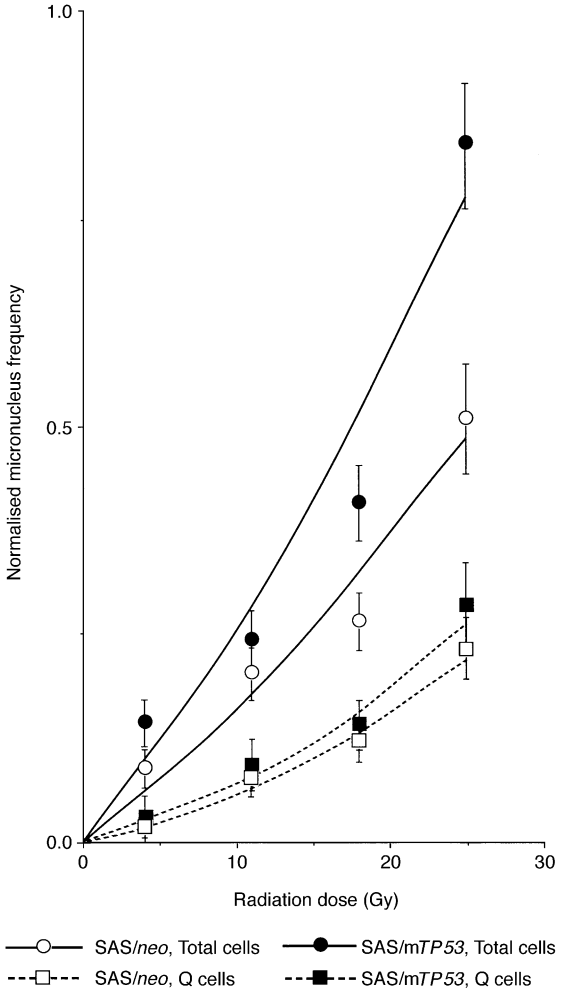

Fig. 4. Normalised micronucleus frequencies for total (circles) and quiescent (Q) (squares) cells of SAS cells transfected with *neo* vector (SAS/*neo*) (open symbols) or SAS cells transfected with mutant p53 (SAS/m*TP53*) (solid symbols) tumour cell lines following γ-ray irradiation. Bars represent 95% Confidence Limits (CL).

Table 2
Dose modifying factors for quiescent cells relative to the total tumour cell populations[a]

End-point	SAS/*neo*	SAS/m*TP53*
Normalised apoptosis frequency[b]		
0.1	2.2 (2.0–2.4)[c]	–
0.05	2.6 (2.3–2.9)	–
0.03	2.7 (2.4–3.0)	3.3 (2.9–3.7)
Normalised micronucleus frequency[d]		
0.25	–	2.4 (2.2–2.6)
0.2	2.1 (1.9–2.3)	2.6 (2.3–2.9)
0.1	2.8 (2.4–3.2)	3.6 (3.2–4.0)
0.05	3.0 (2.5–3.5)	4.0 (3.4–4.6)

[a] The ratio of γ-ray dose needed to obtain each end-point in quiescent tumour cells and γ-ray dose needed to obtain each end-point in total tumour cells.

[b,d] Normalised frequency = the frequency minus the frequency in the non-irradiated tumours.

[c] Numbers in parentheses are 95% Confidence Limits (CL), determined using mean values, standard deviations, and the numbers of observations on which the means and standard deviations were based.

the SF determined for total tumour cells, the HF of Q cells was 43.3% (38.8–46.8%) for SAS/*neo* and 54.6% (49.1–60.1%) for SAS/m*TP53* tumours. Meanwhile, based on the SFs of Q cells extrapolated using the values of the normalised MN frequency of Q cells and the regression lines for the relationship between the normalised MN frequency and the SF determined for total tumour cells, the HF of Q cells was 45.8% (41.1–50.5%) for SAS/*neo* and 48.8% (43.5–54.1%) for SAS/m*TP53* tumours.

4. Discussion

The contribution of apoptosis to the response of tumours depends on the cell type [15,25], as well as the induction of micronucleation after DNA-damaging treatment [26]. Namely, the ratio of apoptosis or micronucleation to total cell death depends on the cell type [16,26]. The time course of the change in the apoptosis frequency (Fig. 1) showed that death by

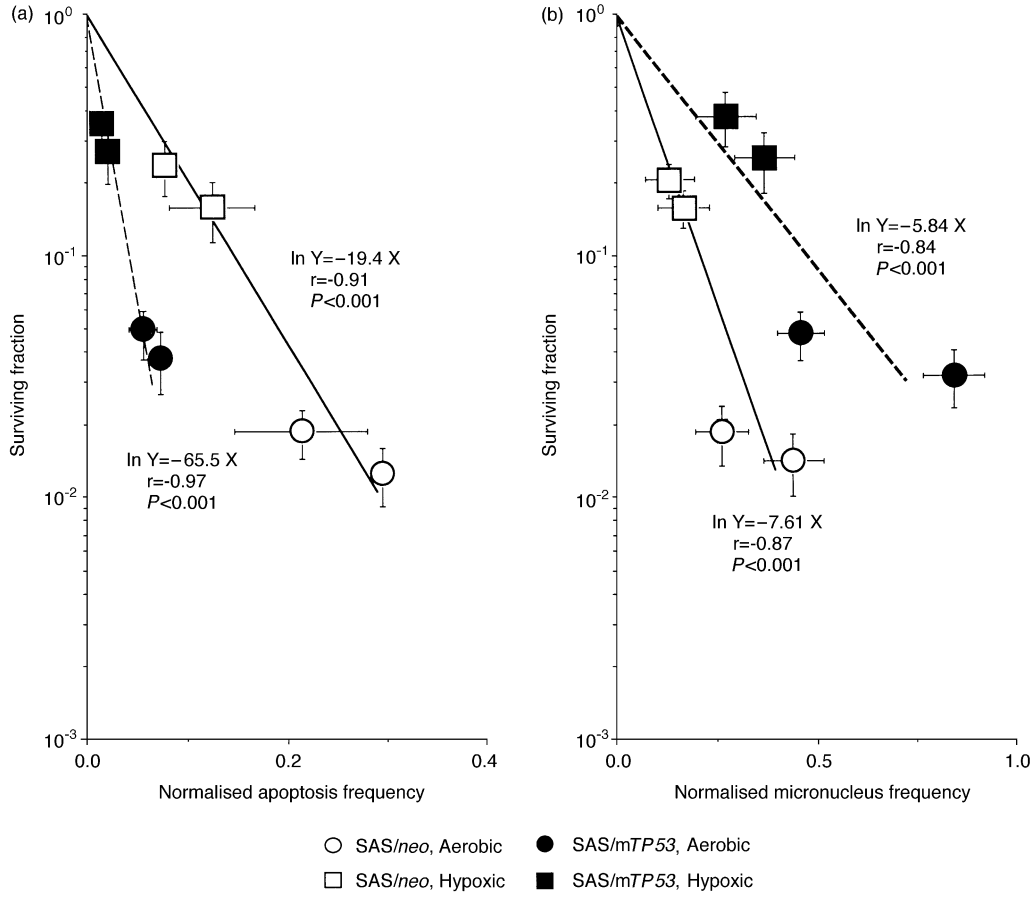

Fig. 5. Correlations between the normalised apoptosis frequency and the surviving fraction (SF) (a) and between the normalised micronucleus frequency and the SF (b) for total tumour cell populations of SAS cells transfected with *neo* vector (SAS/*neo*) (open symbols) or SAS cells transfected with mutant p53 (SAS/m*TP53*) (closed symbols) tumours. Tumour-bearing mice were irradiated while alive (circles) or after clamping the proximal end of the tumours to make them totally hypoxic (squares). Bars represent 95% Confidence Limits (CL).

apoptosis was closely related to the response to γ-ray radiation in SAS/*neo* tumours but not in the SAS/m*TP53* tumours. However, whichever method was used, in both tumour cells, the apoptosis frequency showed a similar profile and a maximum value at 6 h after irradiation. Thus, as far as these two tumour cells are concerned, the fluorescence staining method was thought to reflect apoptotic events as well as the conventional TUNEL method. In addition, as shown in Fig. 5, since the correlation between apoptosis frequency and SF and between MN frequency and SF in total cells of both tumours had significant linearity ($P<0.001$), both the apoptosis frequency and MN frequency are indices that reflect the sensitivity of these two tumour cells.

Fig. 2 shows that SAS/m*TP53* tumour cells within solid tumours with a diameter of 7 mm are less radiosensitive to γ-rays in terms of clonogenic survival than SAS/*neo* tumour cells. This is quite consistent with reports that tumour cells with a mutant *TP53* gene were more radioresistant than those with a wild *TP53* gene [20,27]. In addition, at doses above that high enough to kill almost all intratumour aerobic cells, the slopes of both survival curves were starting to plateau out and were nearly parallel. This was thought to reflect the dose-survival curves of the hypoxic cells within these two solid tumours in the range and that the sensitivity to γ-rays of these two hypoxic tumour cells was similar.

As shown in Figs. 3 and 4, as a whole, Q cells showed significantly lower radiosensitivity than total cells ($P<0.05$) in both tumours. In addition, whichever normalised frequency was employed as an end-point for the radiation effect, the difference in radiosensitivity between total and Q cells was significant ($P<0.05$). That is to say, the radiosensitivity of Q cells was invariably significantly lower than that of total cells ($P<0.05$) (Table 2). This is probably because γ-ray irradiation produced less apoptosis-inducing DNA damage under hypoxic conditions than under aerobic conditions. In contrast, at 0 Gy, Q cells and hypoxic cells tended to indicate higher apoptosis frequencies than total cells and aerobic cells, respectively (Table 1). This was presumably due, in part, to the effect of hypoxia-induced apoptosis [28]. Meanwhile, when tumours were irradiated

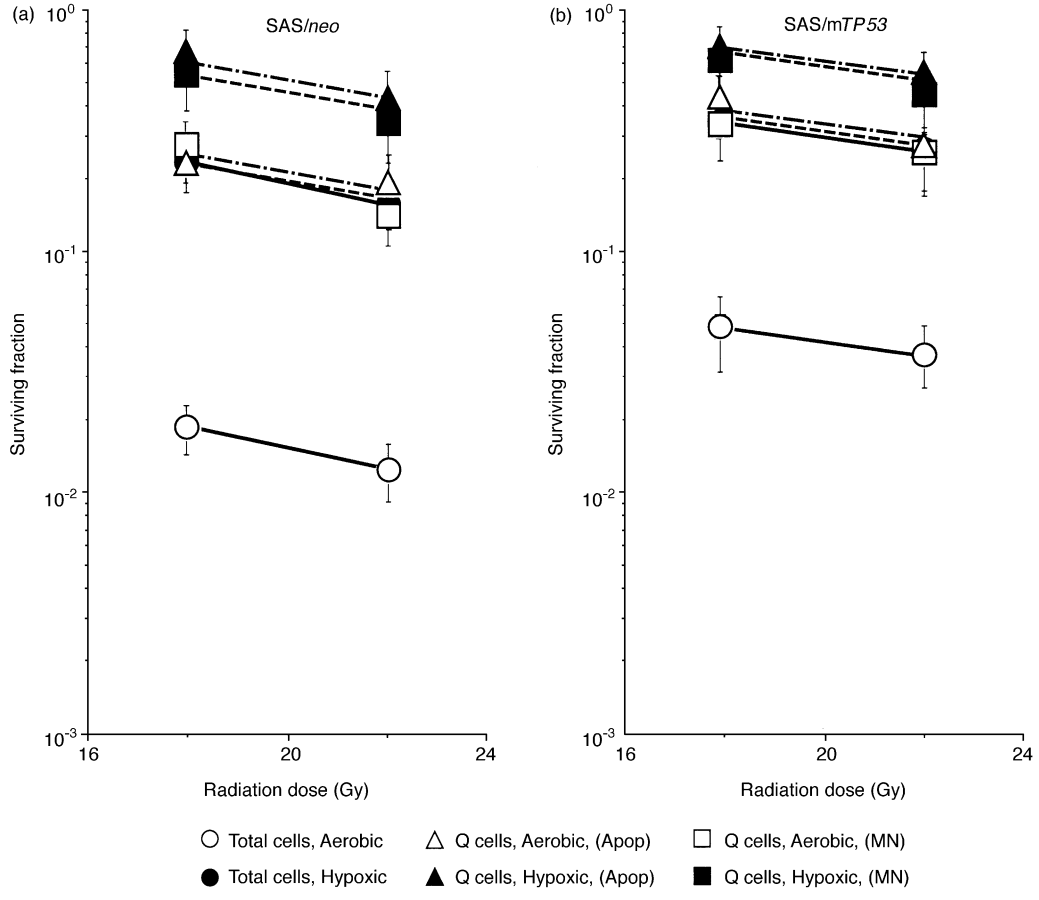

Fig. 6. Cell survival curves for total cell populations (circles) and quiescent (Q) cells (triangles and squares) of SAS cells transfected with *neo* vector (SAS/*neo*) (a) and SAS cells transfected with mutant p53 (SAS/m*TP53*) (b) tumours. The cell survival curves for Q cells were based on the data concerning apoptosis (Apop) frequency (triangles) or micronucleus (MN) frequency (squares). Tumours irradiated in mice while alive or after clamping of the proximal end of tumours to make them totally hypoxic are shown by open and solid symbols, respectively. Bars represent 95% Confidence Limits (CL).

with γ-rays, SAS/*neo* was significantly more susceptible to apoptosis in both total and Q cells ($P<0.05$), and SAS/m*TP53* was significantly more susceptible to micronucleation in total cells ($P<0.05$) and, although not significantly, more susceptible in Q cells. This is probably because radiation-induced p53-dependent apoptosis was almost completely abolished in the SAS/m*TP53* tumour cells due to the loss of function of the mutant p53 protein, resulting in an increase to some extent in micronucleation following irradiation. However, this increase could not supplement the decrease in cell death induced by apoptosis. As a result, SAS/m*TP53* tumour cells were thought to be more radioresistant than SAS/*neo* tumour cells in terms of clonogenic survival. However, since the p53-independent apoptotic pathway was not damaged, the dose–response curves of apoptosis frequency were obtained even for the SAS/m*TP53* cells. Actually, a p53-independent apoptotic pathway is already known [29], and it is claimed that the human promyelocytic leukaemia cell line, HL-60, which is known to be deficient in p53 because of large deletions, can undergo apoptosis following X-ray irradiation [30]. Moreover, there is a report that dose–response curves of apoptosis were seen in Chinese hamster V79 cells bearing a mutated *TP53* following high linear energy transfer carbon-ion beam irradiation [31].

As mentioned above, significant linearity ($P<0.001$) between the normalised apoptosis frequency and SF and between the normalised MN frequency and SF demonstrated the usefulness of both these frequencies like clonogenic survival as an index for radiation-induced tumour cell death (Fig. 5). However, the number of apoptotic tumour cells clearly increased with the decrease in SF in SAS/*neo*, but not for SAS/m*TP53*. In contrast, the decrease in SF caused an increase in the MN frequency in SAS/m*TP53*, but not so large an increase in SAS/*neo*. In general, the clonogenic survival assay reflects all modes of cell death. Accordingly, this means that the ratio of apoptosis to total cell death was large and small, and that of micronucleation was small and large, in the SAS/*neo* and SAS/m*TP53* tumour cells, respectively.

The survival curves and their slopes in Fig. 6 confirmed that Q tumour cells were more radioresistant than total cells whether aerobic or hypoxic. The sizes of the HF calculated from the best paired survival curves showed that the Q cells had significantly larger HFs than the total cells ($P<0.05$) in both tumours and that the SAS/m*TP53* tumour had a significantly larger HF than the SAS/*neo* tumour in the total tumour cell populations ($P<0.05$) and, although not significantly, also had a larger HF in the Q tumour cell populations. Meanwhile, the SAS/m*TP53* tumour showed a significantly lower value of cell yield after mincing and trypsinisation ($P<0.05$) and again, although not significantly, had a lower value of LI after 7 days continuous labelling with BrdU than the SAS/*neo* tumour. This implies that SAS/m*TP53* cells are more tightly connected to one another than SAS/*neo* cells in solid tumours and that the SAS/m*TP53* tumour comprises a larger hypoxic cell-rich Q cell fraction than the SAS/*neo* tumour. The latter finding was consistent with the fact that total cells in SAS/m*TP53* tumours included larger HFs than those in SAS/*neo* tumours. However, in our recent study using four different tumour cell lines, it was shown that no apparent relationship exists between the size of the HF and the value of cell yield [16]. Accordingly, it is hard to consider that the difference in cell yield was related to that in the size of the HF in Q cells. In future, further histopathological study may be needed.

There is also a report that mutant p53 potentiates the induction by protein kinase C of the synthesis of vascular endothelial growth factor which may result in increased angiogenesis [32]. Thus, increased blood supply may facilitate the development and proliferation of an abnormal clone with mutant p53. Actually, it was reported that SAS/m*TP53* solid tumours grew more rapidly than SAS/*neo* tumours [33]. Essentially, in SAS/m*TP53* solid tumours, cells with mutant p53 could escape from the checkpoint mechanism for maintaining genomic stability by wild-type p53, and proliferate without cell cycle arrest or apoptosis. Furthermore, the increased blood supply due to angiogenesis could facilitate tumour proliferation. However, as is often the case with advanced malignant solid tumours, in due course, neovascularisation by angiogenesis could not catch up with the rapid tumour cell proliferation, resulting in a large HF and Q cell fraction in solid tumours.

Solid tumours, especially human tumours, are thought to contain a high proportion of Q cells [10]. The presence of these cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumour core, and this is another consequence of poor vascular supply [10]. This might promote MN formation and apoptosis in Q tumour cells, partly due to the effect of hypoxia-induced apoptosis (Table 1) [28]. It has been reported that Q cells have lower radiosensitivity than P cells in solid tumours *in vivo* [10,12,16,34]. This means that more Q cells survive after radiotherapy than P cells. Consequently, the control of Q cells has a great impact on the outcome of anticancer radiation therapy. However, as shown in Table 2, the difference in sensitivity between total and Q cells did not depend on the p53 status of tumour cells. Thus, from the viewpoint of the tumour cell killing effect including intratumour Q cell control, a treatment modality for enhancing the Q cell response has to be considered. Our previous studies using mouse tumour cells with wild-type p53 status have shown that combined treatment with tirapazamine (SR-4233, WIN 59075, 3-amino-1,2,4-benzotriazine-1,4-dioxide), a well-known bioreductive agent, and/or heat

treatment at mild temperatures in conjunction with DNA damaging treatment such as radiotherapy or chemotherapy is effective in terms of enhancing the intratumour Q cell response [34–36]. In future, we would like to examine whether the effects of these treatments on intratumour Q cells depends on the p53 status of the cells.

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References

1. El-Deiry WS, Tokino T, Velculescu VE, *et al*. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993, **75**, 817–825.
2. Dulic V, Kaufman W, Wilson S, *et al*. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* 1994, **76**, 1013–1023.
3. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci, USA* 1995, **92**, 8493–8497.
4. Kastan M, Zhan Q, El-Deiry WS, *et al*. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 1992, **71**, 587–597.
5. Mummenbrauer T, Janus F, Muller B, Wiesmuller L, Deppert W, Grosse F. p53 protein exhibits 3′-to-5′ exonuclease activity. *Cell* 1996, **85**, 1089–1099.
6. Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature* 1994, **370**, 220–223.
7. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995, **80**, 293–299.
8. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991, **351**, 435–456.
9. Lowe SW. Cancer therapy and p53. *Current Opinion in Oncology* 1995, **7**, 547–553.
10. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 1998, **58**, 1408–1416.
11. Siemann DW. The tumor microenvironment: a double-edged sword. *Int J Radiat Oncol Biol Phys* 1998, **42**, 697–699.
12. Masunaga S, Ono K, Abe M. A method for selective measurement of the radiosensitivity of quiescent cells in solid tumors—combination of immunofluorescence staining to BrdU and micronucleus assay. *Radiat Res* 1991, **125**, 243–247.
13. Miller BM, Werner T, Weier H-U, Nusse M. Analysis of radiation-induced micronuclei by fluorescence in situ hybridization (FISH) simultaneously using telomeric and centromeric DNA probes. *Radiat Res* 1992, **131**, 177–185.
14. Fenech M, Denham J, Francis W, Morley A. Micronuclei in cytokinesis-blocked lymphocytes of cancer patients following fractionated partial-body radiotherapy. *Int J Radiat Biol* 1990, **57**, 373–383.
15. Meyn RE, Stephens LC, Ang KK, *et al*. Heterogeneity in the development of apoptosis in irradiated murine tumours of different histologies. *Int J Radiat Biol* 1993, **64**, 583–591.
16. Masunaga S, Ono K, Suzuki M, Kinashi Y, Takagaki M. Radiobiologic significance of apoptosis and micronucleation in quiescent cells within solid tumors following γ-ray irradiation. *Int J Radiat Oncol Biol Phys* 2001, **49**, 1361–1368.
17. Ota I, Ohnishi K, Takahashi A, Yane K, *et al*. Transfection with mutant p53 gene inhibits heat-induced apoptosis in a head and neck cell line of human squamous cell carcinoma. *Int J Radiat Oncol Biol Phys* 2000, **47**, 495–501.
18. Takahashi A. Different inducibility of radiation- or heat-induced p53-dependent apoptosis after acute or chronic irradiation in human cultured squamous cell carcinoma cells. *Int J Radiat Biol* 2001, **77**, 215–224.
19. Jia LQ, Osada M, Ishioka C, *et al*. Screening the p53 status of human cell lines using a yeast functional assay. *Mol Carcinog* 1997, **19**, 243–253.
20. Ohnishi K, Wang X, Takahashi A, Ohnishi T. Contribution of protein kinase C to p53 dependent WAF1 induction pathway after heat treatment in human glioblastoma cell lines. *Exp Cell Res* 1998, **238**, 399–406.
21. Stephens LC, Ang KK, Schultheiss TE, Milas L, Meyn RE. Apoptosis in irradiated murine tumors. *Radiat Res* 1991, **127**, 308–316.
22. Thompson CB. Apoptosis in the phathogenesis and treatment of disease. *Science* 1995, **267**, 1456–1462.
23. Ando K, Koike S, Ohira C, *et al*. Accelerated reoxygenation of a murine fibrosarcoma after carbon-ion radiation. *Int J Radiat Biol* 1999, **75**, 505–512.
24. Moulder JE, Rockwell S. Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. *Int J Radiat Oncol Biol Phys* 1984, **10**, 695–712.
25. Hendry JH, West CW. Apoptosis and mitotic cell death: their relative contributions to normal-tissue and tumour radiation response. *Int J Radiat Biol* 1997, **71**, 709–719.
26. Guo GZ, Sasai K, Oya N, *et al*. Simultaneous evaluation of radiation-indiced apoptosis and micronuclei in five cell lines. *Int J Radiat Biol* 1998, **73**, 297–302.
27. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* 1999, **59**, 1391–1399.
28. Minet E, Michel G, Remacle J, Michiels C. Role of HIF-1 as a transcription factor involved in embryonic development, cancer progression and apoptosis (review). *Int J Mol Med* 2000, **5**, 253–259.
29. Han Z, Chatterjee D, He D, *et al*. Evidence for a G2 checkpoint in p53-independent apoptosis induction by X-irradiation. *Mol Cell Biol* 1995, **15**, 5849–5857.
30. Witenberg B, Kletter Y, Kalir HH, *et al*. Ascorbic acid inhibits apoptosis induced by X irradiation in HL60 myeloid leukemia cells. *Radiat Res* 1999, **152**, 468–478.
31. Aoki M, Furusawa Y, Yamada T. LET dependency of heavy-ion induced apoptosis in V79 cells. *J Radiat Res* 2000, **41**, 163–175.
32. Kieser A, Weich HA, Brandner G, Marme D, Kolch W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 1994, **9**, 963–969.
33. Tamamoto T, Takahashi A, Ohnishi K, Ohnishi T. Heat-induced growth inhibition and apoptosis in transplanted human tongue carcinomas with diffrent p53 genes. In Tanaka R, ed. *Advances in Hyperthermic Oncology 2000*. Niigata, Japan, KOKO-DO, 2001, 63–67.
34. Masunaga S, Ono K, Nishimura Y, *et al*. Combined effects of tirapazamine and mild hyperthermia on anti-angiogenic agent (TNP-470) treated tumors—reference to the effect on intratumor quiescent cells. *Int J Radiat Oncol Biol Phys* 2000, **47**, 799–807.
35. Masunaga S, Ono K, Hori H, *et al*. Modification of tirapazamine-induced cytotoxicity in combination with mild Hyperthermia and/or nicotinamide: reference to effect on quiescent tumour cells. *Int J Hyperthermia* 1999, **15**, 7–16.
36. Masunaga S, Ono K, Suzuki M, *et al*. Usefulness of tirapazamine as a combined agent in chemoradiation and thermo-chemoradiation therapy at mild temperatures—reference to the effect on intratumor quiescent cells. *Jpn J Cancer Res* 2000, **91**, 566–572.