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Relationship between growth fraction and clonogenic survival after ionizing irradiation in pancreatic MiaPaCa2 cells

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Background. The prediction of cell radiosensitivity still remains a matter of great interest in the field of radiation biology and oncology.

The aim of the present study was to examine the relationship between the value of growth fraction (GF) prior to irradiation and the clonogenic survival of the pancreatic cancer cell line MiaPaCa2.

Materials and methods. Growth fraction prior to irradiation was determined by a novel method using bromodeoxyuridine-flow cytometry data from a single sample. The cells were irradiated with 2 and 8 Gy using a ^{60}Co source. Cell survival after ionizing radiation was measured using a clonogenic assay. Pearson' product–moment correlation coefficient was used to estimate the correlation of GF and clonogenic survival fraction values.

Results. A positive correlation between GF values and cell survival fraction was obtained after irradiation with 8 Gy ($r = 0.67$, $P = 0.0002$). We observed a trend of a higher GF and better clonogenic survival of cells after irradiation with 2 Gy.

Conclusions. A higher growth fraction prior to irradiation is related to a better clonogenic survival of the pancreatic cell line MiaPaCa2. Whether or not this finding is applicable to other cell types remains to be established.

Key words: MiaPaCa2, growth fraction, irradiation, clonogenic survival

INTRODUCTION

The prediction of cellular radiosensitivity still remains a matter of great interest in the field of radiation biology and oncology. There have been some attempts to identify factors predicting response to radiotherapy (1, 2). Among others, most widely used are the GF value as a proliferation marker and clonogenic cell survival.

However, clonogenic assay is time-consuming and requires extensive experience in performing the microscopic evaluation (3, 4).

GF is the proportion of the cells committed to the cycle (5) and at present is being widely estimated by the immunohistochemical assessment of the nuclear antigen Ki-67 (6–8). The predictive usefulness of GF has been evaluated in a few studies in patients with various tumors, but the results are controversial. Some studies reported that a higher GF is associated with a better response to treatment (9, 10), whereas other studies found no such association (11–14). Discrepancies may depend on various factors such as the lack of standardized methods of assessing GF, different therapies applied to patients (5), a risk of misclassification of the GF marker Ki-67 (15).

These limitations have stimulated interest in developing a new assay *in vitro*. A new method avoiding the drawbacks of proliferative markers and allowing estimation of GF from a single sample after labeling with bromodeoxyuridine (BrdUrd) has recently been described by our group (16).

The aim of the present study was to evaluate the feasibility of this new method for studying the relationship between cell proliferation and cell survival after irradiation. The pancreatic cancer cell line MiaPaCa2 was used for this purpose.

MATERIALS AND METHODS

Cell culture

The pancreatic cancer cell line MiaPaCa2 was obtained from the European Collection of Cell Cultures, human source (Sigma-Aldrich Chemie GmbH). Cells were cultured in DMEM supplemented with 15% of fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% of CO_2.

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Growth fraction determination

Cells were harvested from exponential phase culture by trypsinization, counted and evaluated for viability. To obtain different GF values, the cells were seeded at the plating densities of 0.5–1 × 10^5 cells/cm^2 inside 6-well culture dishes with 2.7-mL of medium containing 15% FCS and incubated at 37 °C in a humidified atmosphere with 5% CO_2 in air. The cell density and GF were determined at 24 and 48 h. For *in vitro* labeling of MiaPaCa2 cells before ionizing irradiation, BrdUrd was dissolved in phosphate-buffered saline (PBS) and added to culture medium at a final concentration of 10 μM. After incubation for 30 min at 37 °C, the cells were rinsed twice with RPMI 1640 medium, and the culture was further incubated at 37 °C. Samples for analysis were taken 12 h after the BrdU pulse, the cells were trypsinized, resuspended in medium, washed with PBS, fixed in 70% ice-cold ethanol and stored at +4 °C until staining.

Suspensions of fixed MiaPaCa2 cells were twice washed in PBS and incubated in 2 N HCl / Triton X-100 0.5% for 30 min at room temperature for DNA denaturation. The acid solution was then neutralised with 0.1% $Na_2B_4O_7$ and the cells were washed in PBS; 50 μl of PBS and 20 μl of FITC-conjugated mouse anti-BrdUrd monoclonal antibody was added to the pellet containing 106 nuclei and incubated for 1 h at room temperature in the dark. After washing in PBS, 1 ml of a solution containing 5 mg/l propidium iodide in PBS was added to the pellet and incubated for 30 min at 37 °C in the dark.

The cellular DNA content and the amount of incorporated BrdUrd were simultaneously measured using a FACSort flow cytometer (Becton Dickinson). Data from 10000 nuclei per sample were acquired as dot plots of BrdUrd labeling vs. DNA content and analysed using CellQuest software (Becton Dickinson). GF was calculated from bromodeoxyuridine-flow cytometry data using WinMDI software version 2.8 as described in previous studies at our laboratory (16).

Cell irradiation

After 24 and 48 h of culturing, the cells were irradiated with a dose of 2 or 8 Gy at room temperature using a ^{60}Co source. Control wells (0 Gy) were left in the radiation control room during the radiation session.

Clonogenic survival

Clonogenic survival was defined as the ability of cells to maintain clonogenic capacity and to form colonies. For the clonogenic assay, immediately after irradiation, MiaPaCa2 cells were trypsinized, resuspended in medium and seeded in due densities of cells per well in triplicate into 35 mm dishes. Cells were incubated for 14 days at 37 °C in a humidified atmosphere containing 5% CO_2, then washed with PBS, fixed and stained in 1.0% crystal violet in ethanol. As a colony is defined as consisting of at least 50 cells (17), only colonies having more than 50 cells were evaluated by light microscopy and scored as surviving. The survival fraction (SF) was calculated by the equation: SF = colonies counted / cells seeded × PE (plating efficiency) / 100. Plating efficiency was defined as the colony number divided by the number of cells seeded × 100% (18).

Statistical analysis

Triplicate determinations were made within each of the four independent experiments. Statistical differences between the groups were analysed by Student's t test. Pearson's product-moment correlation coefficient was used to estimate the correlation of GF and clonogenic survival fraction values. A value of $P < 0.05$ was considered significant.

RESULTS

Assuming that GF increases with increasing cell densities during the exponential growth phase, we expected to obtain different GF values in the same experiment prior to irradiation at 24 and 48 h of cell culturing. The values of GF and cell densities at 24 and 48 h in four different experiments are shown in Fig. 1. This figure shows that, in contrast to our assumption, although cell densities in each separate experiment increased about twofold, the GF values for 24 to 48 h remained stable. The mean values of GF of each independ-

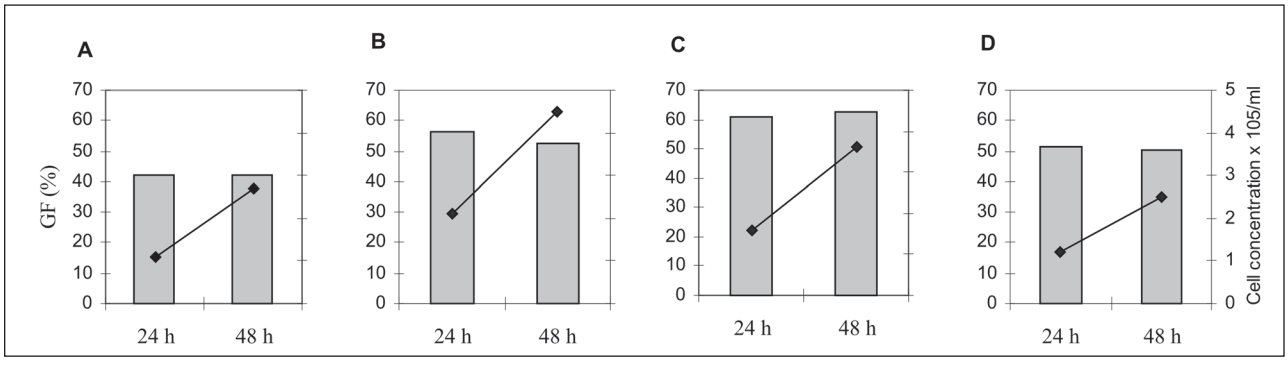

Fig. 1. Growth curves and GF of MiaPaCa2 cells prior to irradiation in four independent experiments (A, B, C and D). The number of viable cells and GF were evaluated at 24 and 48 h after seeding. Bars represent GF values. Lines represent cell densities / cm^2 × 10^5. Mean values of triplicate wells are shown

ent experiment were 42 ± 1%, 62 ± 3%, and 54 ± 3%, and 51 ± 1%. Dependence between GF and cell densities was obtained only at 24 h (Fig. 2).

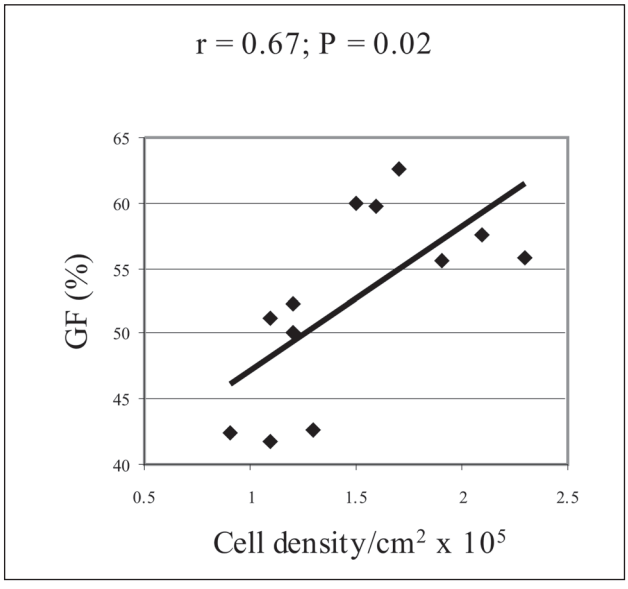

Fig. 2. Correlation between GF values and cell densities after 24 h of culturing. Data points show the values of each measurement

To investigate the radiation response of MiaPaCa2 cells, cultures were irradiated with 2 and 8 Gy doses of radiation, and clonogenic assays were estimated after 14 days of culturing. The survival of MiaPaCa2 cells varied depending on irradiation dose. The mean SF2 of four independent experiments was 0.27 ± 0.06 (range. 0.14–0.39). The mean SF8 was 0.009 ± 0.003 (range, 0.001–0.029).

To determine whether there is an association between GF values before irradiation and cell survival after irradiation, the surviving fractions were plotted against GF values (Fig. 3). The Pearson correlation coefficient r was calculated to measure the correlating behaviour among different markers. Although we hypothesized that a higher GF may correlate with a better response to irradiation and thus in part lead to a poor clonogenic survival, we observed a positive statistical correlation between GF values and cell surviving fraction only after irradiation with 8 Gy. A trend for a better clonogenic survival of cells with a higher GF before irradiation was obtained after 2 Gy irradiation. The lack of statistically significant correlation between GF and SF2 encourages a future parallel assessment of GF and clonogenic survival for other cell types.

DISCUSSION

Whether the GF assay can be used as a predictive tool to estimate the intrinsic cellular radiosensitivity depends on whether the results correlate with clonogenic survival. In this study, a comparison was made between two potential methods of studying radiosensitivity: GF determined by a novel method using bromodeoxyuridine-flow cytometry data from a single sample and a clonogenic assay. Traditionally, the clonogenic assay has been considered to be the optimal assay method for determining survival after radiation (19). It relies on the ability of cells to form viable colonies derived from a single "clone". There is very little and conflicting data about the predictive value of GF in clinical studies and almost no data on the correlation with clonogenic survival in vitro.

Tumors with a higher GF show a higher response to radiotherapeutic treatment (20). Therefore, one could expect that high GF should predict a poor clonogenic survival of irradiated cells. In contrast, we observed the opposite relationship. A trend was apparent where the higher GF was associated with a better survival of cells after ionizing irradiation.

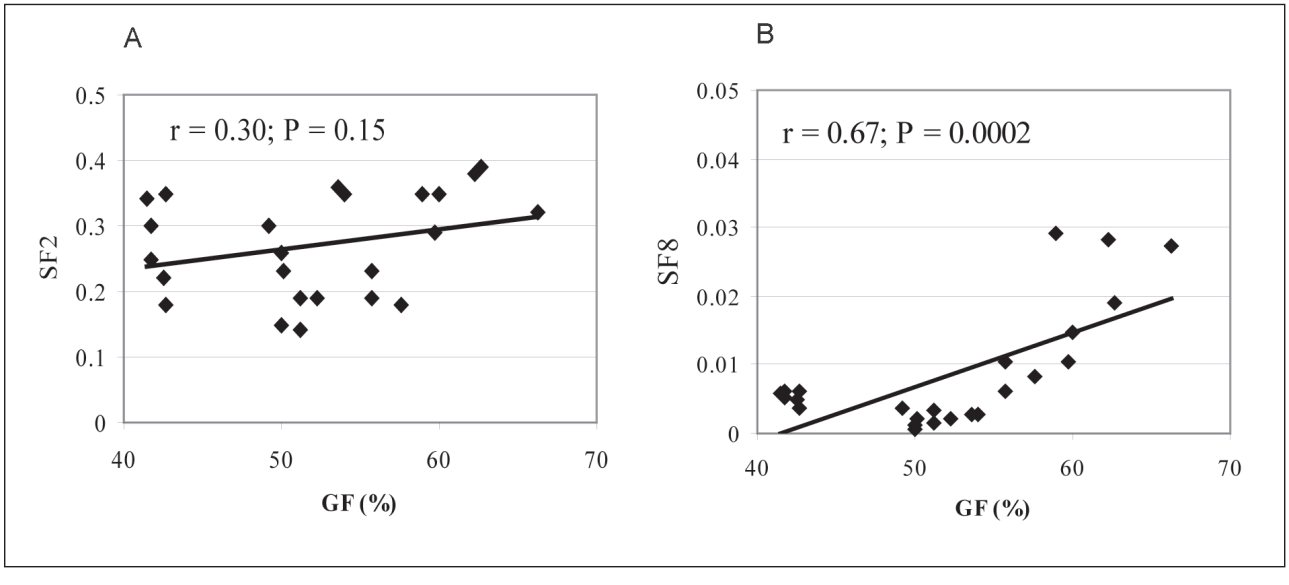

Fig. 3. Correlation between GF values prior to irradiation and surviving fraction after irradiation with 2 Gy (A) and 8 Gy (B). Colonies were counted 14 days post irradiation. Data points show the values of each measurement. SF2 and SF8 denote surviving fractions at 2 Gy and 8 Gy

As for different correlations between GF and clonogenic survival at 2 and 8 Gy, some hypotheses are involved. One of them concerns different molecular mechanisms activated by different irradiation doses. Marekova et al. (21) have demonstrated that irradiation of cells induces two types of cell death in a dose-dependent manner where after higher doses cells die by apoptosis at all phases of the cell cycle, whereas after lower doses, cells die by apoptosis after G2 / M arrest or an attempt of mitosis.

The other hypothesis is cancer stem cell theory. The existence of cancer stem cells within human pancreatic adenocarcinomas and in established pancreatic carcinoma cell lines has recently been proven (22–24). Cancer-initiating cells may be intrinsically radioresistent (25).

We suppose that MiaPaCa2 cells and stem cells in culture exhibit different levels of radiosensitivity. Consequently, actively proliferating cells may be killed by irradiation, but stem cells may survive and repopulate. A higher GF in cultures after treatment with a single dose of 2 Gy has been described in primary cultures of head and neck tumours (26). Future work will be needed to perform the phenotypic characterization that may help define cell subpopulations responsible for repopulation.

CONCLUSIONS

Our data show that a higher growth fraction prior to irradiation is related with a better clonogenic survival of the pancreatic cell line MiaPaCa2. Whether or not this finding can be applied to other cell types remains to be established.

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KASOS MiaPaCa2 LĄSTELIŲ AUGIMO FRAKCIJOS SĄSAJA SU KLONOGENINIU IŠGYVENIMU PO JONIZUOJANČIOSIOS SPINDULIUOTĖS

Santrauka

Įvadas. Ląstelių radiojautrumo įvertinimas iki šiol yra aktualus radiobiologijoje ir onkologijoje. Šio darbo tikslas – ištirti kasos vėžio MiaPaCa2 ląstelių linijos augimo frakcijos (AF) sąsają su klonogeniniu išgyvenimu po jonizuojančiosios spinduliuotės.

Priemonės ir metodai. AF prieš spinduliuotę nustatyta nauju BrdU įjungimo-tėkmės citometrijos metodu. Ląstelės apšvitintos ^{60}Co šaltinio vienkartinėmis 2 ir 8 Gy dozėmis. Ląstelių išgyvenimas po jonizuojančiosios spinduliuotės nustatytas kolonijų formavimo metodu. GF ir klonogeninio išgyvenimo ryšio stiprumas įvertintas Pirsono koreliacijos koeficientu (r).

Rezultatai. Nustatyta teigiama koreliacija ($r = 0,67$, $P = 0,0002$) tarp AF ir klonogeninio išgyvenimo po 8 Gy jonizuojančiosios spinduliuotės. Teigiamos koreliacijos tendencija pastebėta ir po 2 Gy spinduliuotės.

Išvados. Didesnė kasos vėžio MiaPaCa2 ląstelių linijos augimo frakcija prieš spinduliuotę yra susijusi su geresniu klonogeniniu išgyvenimu. Tolesniais tyrimais bus nustatoma AF ir klonogeninio išgyvenimo sąsaja kitose ląstelių linijose.

Raktažodžiai: MiaPaCa2, augimo frakcija, spinduliuotė, klonogeninis išgyvenimas