A Study on Differences between Radiation-induced Micronuclei and Apoptosis of Lymphocytes in Breast Cancer Patients after Radiotherapy

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Z. Naturforsch. **60 c**, 938–942 (2005); received December 28, 2004/May 9, 2005

Cancer patients' responses to radiotherapy vary in severity. It has been suggested that it may be due to differences in intrinsic cellular radiosensitivity. Prediction of tissue reactions to radiotherapy would permit tailoring of dosage to each patient. Towards this goal the micronucleus and apoptosis tests have been proposed as methods for measurement of chromosomal damage in peripheral blood lymphocytes. In this study, gamma-ray sensitivity of cultured lymphocytes of 26 breast cancer patients with early or late reactions was investigated. After irradiation with 4 Gy gamma radiation in G_0, the frequency of micronuclei for patients with early reactions was significantly higher ($P < 0.05$) than for patients with late reactions. In the contrary the frequency of apoptosis for patients with early reactions was significantly lower ($P < 0.05$) than in the other group. It could be suggested that such a reduced amount of micronuclei in the late effects group is due to the presence of some residual DNA damages which are not completely repaired and lesions show increasing severity when the patients' cells are irradiated again. These induced damages, probably are high enough to stimulate other endpoints like apoptosis instead of micronuclei.

Key words: Breast Cancer, DNA Damage, Radiosensitivity

Introduction

Cancer patients' responses to radiotherapy vary in severity (Tucker *et al.*, 1992; Turesson *et al.*, 1996). A range of normal tissue reactions is seen in patients, from very mild to extremely severe and occasionally lethal (Turesson and Thames, 1989). A major determinant of normal tissue reaction is the intrinsic radiosensitivity of the cells (Turesson, 1990).

Much of radiobiology is concerned with increasing our understanding of why and how normal tissues and tumors respond to radiation with the ultimate aim of improving cancer treatment by radiotherapy. Hence the need for reliable predictive assays for normal tissues and tumors response to radiation remains a prime objective of clinical oncology.

The micronuclei assay and apoptotic cell death are biological indicators for assessment of radiosensitivity (Muller *et al.*, 1996; Hendry and West, 1997).

The complexity and laboriousness of enumerating aberrations in metaphases has stimulated the development of a simpler system of measuring chromosome damage. Schmid and Heddle proposed independently that measuring micronuclei is an alternative and simpler approach to assess chromosome damage *in vivo* (Schmid, 1975; Heddle, 1973). Micronuclei are discrete round bodies of nuclear origin found in the cytoplasm outside the main nucleus. They are expressed in dividing cells that either contain chromosome breaks lacing centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interface nucleus with the exception that they are smaller than the main nuclei in the cell, hence they are "micronuclei". In 1985 Fenech and Morley found that the addition of cytochalasin B (cyt B) to the culture medium prevents cells from completing the division cycle by inhibiting cytokinesis but not karyokinesis. By using this technique, cells with only one mitotic division since the addition of cyt B included two nuclei (Fenech and Morley, 1985a, b).

0939–5075/2005/1100–0938 $ 06.00

Physiological cell death was described initially based on morphological grounds only, and was given the name apoptosis. Morphological changes at the microscopic level associated with apoptosis are condensation of chromatin into crescent-shaped caps at the nuclear periphery, disintegration of the nuclei, and reduction in nuclear size, shrinkage of total cell volume, budding (blebbing) and constriction of both the nucleus and the cytoplasm into multiple, small, membrane-bound apoptotic bodies (Kerr *et al.*, 1972).

Peripheral blood lymphocytes are ideal cells for measuring normal tissue radiosensitivity, since they are easy to obtain and to use for *in vitro* tests. Lymphocytes are particularly susceptible to DNA damage-induced agents because of their high potential for mutation. They undergo DNA damage in a time- and dose- dependent manner. Because of the kinetics and the relative sensitivity of lymphocytes to low doses of radiation, they may be a useful biological dosimeter for radiation exposure. On the other hand they are a synchronous cell population, so that the synchrony would obviate the difference caused by mixing of cells treated at different stages of the cell cycle (Evans and O'Riordan, 1975; Catena *et al.*, 1996; Boreham *et al.*, 2000). Following the investigations by Jones *et al.* (1995), we compared the sensitivity of lymphocytes of breast cancer patients with early/late reactions after radiotherapy by studying the radiation induced micronuclei and apoptosis frequency. This study could be used as a test to predict clinical radiation reactions.

Materials and Methods

Blood samples

Heparinzed peripheral blood samples were obtained from 26 breast cancer patients with early or late reactions aged between 36 and 72 years. Fifteen patients had shown early tissue damage (erythema, dry desquamation, moist desquamation) and eleven patients had shown late tissue damage (fibrosis, skin telangiectasia, pigmentation). Patient characteristics and treatment parameters are presented in Table I. Lymphocytes were separated from whole blood on Ficoll-Hypaque gradients (AXIS-SFIELD poC AS Oslo, Norway), washed twice in phosphate buffered saline (PBS) and resuspended in RPMI 1640 (Gibbco BRL) medium modified with L-glutamine containing 10% heat-inactivated fetal calf serum (Gibbco BRL), 100 U

Table I. Patient characteristics and treatment parameters.

Number	Age[a]	Path[b]	GD[c]	Days of rad[d]	Days of chem[e]	Side effects
1	50	D[g]	5000	25	8	Late
2	36	LO[h]	5000	25	6	Early
3	44	D	5000	25	3	Late
4	42	LO	5000	25	6	Early
5	48	D	5000	25	6	Early
6	65	D	5000	25	–	Early
7	39	D	5000	25	6	Late
8	46	D	5000	25	6	Early
9	50	D	5000	25	6	Early
10	38	D	5000	25	6	Early
11	62	D	5000	25	6	Early
12	44	D	5000	25	6	Early
13	68	D	5000	25	9	Early
14	43	D	5000	25	6	Early
15	53	D	5000	25	6	Early
16	72	D	5000	25	6	Late
17	53	D	5000	25	–	Late
18	41	D	3000	25	6	Late
19	44	D	5000	25	6	Late
20	51	I[i]	5000	25	–	Early
21	41	D	5000	21	6	Early
22	46	D	5000	30	6	Late
23	62	D	5000	25	6	Early
24	46	D	5000	25	6	Late
25	47	D	5000	25	6	Late
26	71	D	5000	30	2	Late

[a] Age in years at time of blood sampling; [b] histopathological type of tumor; [c] given dose in Rad; [d] number of days that patients were treated in radiotherapy; [e] number of days that patients were treated in chemotherapy; [g] D, ductal; [h] LO, lobular; [i] I, *in situ.*

ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Boehringer Mannheim GmbH, Germany).

Irradiation

Each sample was divided into two parts, one part was used as a control and the other part was irradiated with 4 Gy gamma radiation using a ^{60}Co source (Gamma beam 150 Atomic Energy, Canada).

Micronuclei assay

Samples were kept for 1 h at 37 °C after being irradiated. The cells were then cultured at a concentration of 10^6 cells ml^{-1} at 37 °C in a humidified atmosphere containing 5% CO_2 adding 15 μg ml^{-1} phytohaemagglutinin-M (Boehringer Mannheim GmbH) to stimulate proliferation. After 44 h incubation, 4.0 μg ml^{-1} Cyt-B (Sigma) were added, and cells were harvested at 72 h. The cells were treated with a lypotonic solution containing 0.075 M KCl for 4–6 min, to obtain good preservation of the cytoplasm. Following centrifugation at 16.1 × *g* for 5 min, the cells were fixed in 3:1 (v/v) methanol/acetic acid. Afterwards, the cells were dropped onto cooled slides with a Pasteur pipette and air-dried. The slides were stained in 10% Giemsa (Merck) and the MNs were scored in 1000 binucleate cells. They were observed by a light microscope using a 400x magnification based on the criteria summarized by Fenech (2000).

Apoptosis assay

The cells in three different vials were cultured with a concentration of 0.5 × 10^6 cells ml^{-1} at 37 °C in a humidified atmosphere containing 5% CO_2. The cells of the three vials were harvested at 24, 48 and 72 h. The cells were collected by centrifugation at 16.1 × *g* for 5 min. The cells were preserved using a 5% acetic acid fixative, followed by addition of a mixture of 3:1 (v/v) methanol/acetic acid for 10 min. The cells in suspension were collected by centrifugation (16.1 × *g* for 5 min), and the fixative was removed. Then 0.5 ml of the fixative was used to resuspend the cells pellet and approx. 50 μl were spread onto a microscope slide. The slides were air-dried and stained in 10% Giemsa. The apoptotic cells were scored in 1000 cells with the light microscope using a 1000x magnification.

Statistical analysis

Student's test was performed to assess the differences between experimental groups.

Results

Micronucleus frequency

The measurement of frequency of micronucleated binucleate lymphocytes and the total of micronuclei in 1000 binucleate lymphocytes showed the following results:

Irradiation induced more multimicronuclei in binucleated lymphocytes (tri-quadric- or more micronuclei were formed in irradiated samples).

Irradiation has induced more multimicronuclei in the early group than the late group, suggesting that more sever damage had occurred. The mean of binucleate cells with micronuclei and the mean of the total micronuclei in binucleate cells for patients with early reactions were significantly higher ($P < 0.05$) than for patients with late reactions after irradiation (Table II). In the non-irradiated samples, the mean of binucleate cells containing micronuclei as well as the total of micronuclei in the patients with late reactions were higher than early reaction patients but this difference was not significant.

Detection of apoptotic cells

The results of apoptotic cells counting of patients are graphed in Fig. 1. Irradiation has induced more apoptosis in lymphocytes. In addition the frequency of apoptosis increased with time.

The difference between the mean of apoptotic cells for 24 and 48 h was not significant, but for a

Table II. The mean of the total micronuclei in binucleate cells and binucleated cells with micronuclei for early and late groups.

	0 Gy		4 Gy	
	Total-MN ± Se	Mean binucleated ± Se	Total-MN ± Se	Mean binucleated ± Se
Early group	35.92 ± 6.07	30.75 ± 4.79	383.73 ± 44.06	253.00 ± 25.51
Late group	49.44 ± 21.30	43.89 ± 18.44	258.67 ± 45.70	179.00 ± 27.40

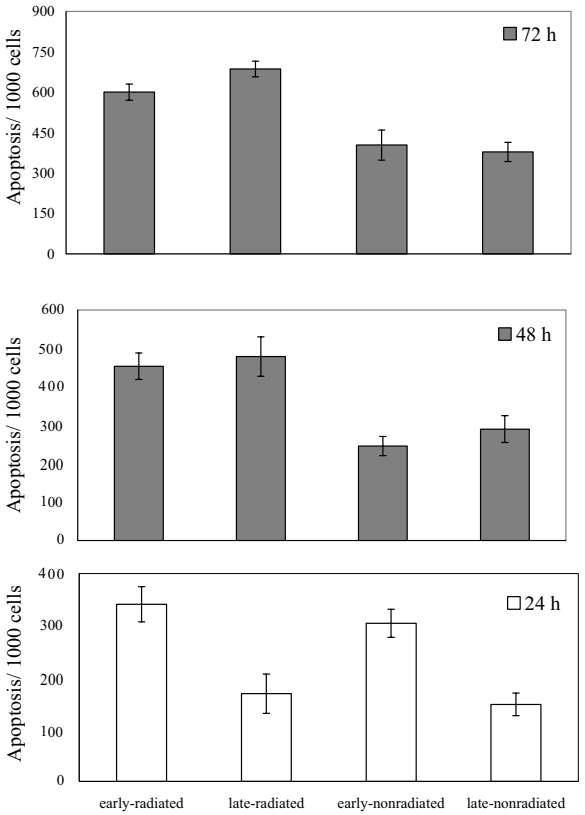

Fig. 1. The frequency of apoptosis in the patients with early and late reaction at 24, 48 and 72 h. The difference between the mean of apoptotic cell for 24 and 48 h were not significant (open bar doted bar, respectively), but for 72 h culture (cross hatched bars) the apoptosis level of patients with late reaction was significantly ($P < 0.05$) lower than the other group.

72 h culture, the apoptosis level of patients with late reaction was significantly ($P < 0.05$) lower than in the other groups (Fig. 1). In the non-irradiated samples, the mean of apoptotic cells in two groups was not significantly different.

Discussion

Using radiation-induced apoptosis and micronucleus formation in lymphocytes of breast cancer patients after radiotherapy as an endpoint, we have compared cellular sensitivity of patients with late reactions and early reactions. Our results showed that cellular sensitivity of the late group is more than of the early group and irradiation can induce sever damages.

Micronucleus formation and apoptosis have been compared previously in several studies attempting to understand the biological relationship between these two indicators of radiation damage (Guo *et al.*, 1998; Belyakov *et al.*, 1999). MN assay and programmed cell death in peripheral blood lymphocytes are useful biodosimeters for estimating the cytogenetic effort after exposure to ionizing irradiation. It is generally assumed that radiation-induced MN and/or apoptosis are on expression cellular radiosensitivity (Fenech *et al.*, 1990; Thierens *et al.*, 1995; Boreham *et al.*, 1996; Cregan *et al.*, 1999). Therefore, radiosensitive cells should have a high frequency of MN/or apoptosis and radioresistant cells should show lower levels. But the relationship between apoptosis and micronucleus formation is not straightforward and it would be simplistic to translate micronucleus frequency into radiosensitivity (Akudugu *et al.*, 2000). After genotoxic damage, a cell may complete its nuclear division and possibly express MN, or may undergo apoptosis (Kirsch-Volders *et al.*, 1997). It is therefore important to evaluate the frequencies of MN and apoptosis cells in estimating the "total" impact of genotoxicity. Then it is probably incorrect to make predictions about relative radiosensitivity based solely on one mode of cell death, and the simultaneous use of the MN assay and the detection of apoptotic cells would be more reliable as a method for predicting cell survival after irradiation (Harms-Ringdahl *et al.*, 1996; Guo *et al.*, 1999).

In a preceding study by Jones and his colleges (Jones *et al.*, 1995) the sensitivity of lymphocytes of breast cancer patients with excessive normal tissue damage after radiotherapy was compared with control group, using chromosome damage. The selection of a suitable control group is very important to catch correct results. Therefore, we have compared two groups of patients. The frequency of MN for patients with early reactions was significantly higher ($P < 0.05$) than for patients with late reactions after irradiation with 4 Gy gamma radiation in G_0. In the contrary the frequency of apoptosis for patients with early reactions was significantly lower ($P < 0.05$) than in the other group.

There were no statistically significant differences in non-radiated samples between the early and late reactions groups for MN and apoptosis.

For the reason of higher cellular sensitivity and probability of residual damages in the late group, the irradiation has induced severe damages. An al-

ternative mechanism of radiation-induced cell killing to that involving chromosome damages is the induction of apoptosis (Wyllie *et al.*, 1980). We can conclude that in the late group, above a certain level of DNA damage in the lymphocytes, lesions that would normally be premitotic apoptosis and hence to low aberration frequencies in mitotic cell.

Acknowledgements

We would like to thank all the blood donors and the personals of Department of Radiotherapy, Cancer Institute, Imam Khomeini Medical Center.

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