PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

WJG, 1999 August; 5(4):365-368
World Journal of Gastroenterology
 ISSN 1007-9327

Micronuclei and cell survival in human liver cancer cells irradiated by 25MeV/u $^{40}Ar^{14+}$ *

LI Wen-Jian[1], GAO Qing-Xiang[2], ZHOU Guang-Ming[1] and WEI Zeng-Quan[1]

Subject headings liver neoplasms; heavy ions; micronucleus; cell survival; tumor cell, cultured

INTRODUCTION

Heavy ions with high linear energy transfer (LET) have the following advantages as compared with the conventional X-rays and γ-rays therapy: a better physical selectivity, a higher relative biological effectiveness (RBE), a greater therapeutic gain factor (TGF), a smaller oxygen enhancement ratio (OER) for LET > 200KeV/μm, positrons emitted from nuclear reaction products offering the condition of monitoring heavy ion position in tissue, and so on.

During the mid 1970s, 450 patients were treated with heavy ions, mostly neon, at the Bevalac of Lawrence Berkeley Laboratory (LBL), United States[1]. Promising results with neon ions were reported when compared with the conventional radiotherapy for soft tissue sarcoma, bone sarcoma and prostate cancer[2,3]. In 1994, the Heavy Ion Medical Accelerator in Chiba (HIMAC) of Japan was designed to deliver beams of ions, helium to argon, to energies in the range of 100MeV/u-800MeV/u. Carbon ions were used for clinical treatment. By August 1997, a total of 301 patients had been treated with carbon ions[4]. The treatment was started at GSI, Darmstadt, Germany in December 1997. Two patients suffe ring from tumors at the base of skull were treated with five and four fractions of carbon ions respectively[5]. In 1995, HIRFL (Heavy Ion Research Facility in Lanzhou, China) began the basic researches of cancer therapy with heavy ions such as carbon, oxygen and argon. In order to collect basic data for clinical therapy, we studied and discussed the dynamic changes of micronuclei and cell survival in human liver cancer cells SMMC-7721 irradiated by 25MeV/u^{40}-Ar $^{14+}$.

[1]Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, Gansu Province, China
[2]Department of Biology, Lanzhou University, Lanzhou 730000, Gansu Province, China
LI Wen-Jian, male, born on 1959-02-22 in Hebei Province, Associate Professor and Deputy Director of the Second Division of Heavy Ion Application. Graduated and obtained a bachelor degree from Hebei University of Technology in 1982, and a master degree from Institute of Modern Physics, Chinese Academy of Sciences in 1992, specialized in the basic study of radiation biological effects and cancer therapy with heavy ions; having 33 papers published.
***Supported by National Climbing Plan Subject of B Category, No.** 85-45-01-3
Correspondence to: LI Wen-Jian, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, Gansu Province, China
Tel. +86·0931·8854897
Received 1999-01-20

MATERIALS AND METHODS

Cells and cell culture

Human liver cancer cells SMMC-7721, purchased from Second Military Medical University in Shanghai, were cultivated in RMPI-1640 medium (Gibco product) supplemented with 10% calf serum in standard incubator at 37℃ in air with 5% CO_2. One passage (subculture) of cells every 6-7 days was performed. The cells were shifted to Φ35mm petri-dishes 2 days before irradiation, each petri-dish had 2mL cell suspension, and density of the cells was 5×10^4 cells/mL. Each dose had 6 petri-dishes. Before irradiation, cells in each petri-dish were examined under reverse light microscope so as to select materials good in growth and even in density.

Selection of ion beams

Irradiation was performed using $^{40}Ar^{14+}$ ion beam with energy of 25MeV/u and intensity of 0.005nA (2.1×10^6p/s). The doses of cells were measured by air ionization chamber. Single (0.68, 6.8 and 68Gy) and fracti onated (twice and thrice, at an interval of 2h, total dose of 68Gy) irradiations were done.

Preparation of samples before irradiation

Four petri-dishes were taken arbitrarily before irradiation, culture suspension, in which adhesive cells were not included, was harvested, and adhesive cells were fixed with Carnoy's fluid. At the same time, two other petri-dishes were taken and cells were harvested by trypsinzation. Cells were stained with typan blue, and number of dead and living cells were counted, respectively. Covers of petri-dishes were removed under condition of asepsis, and irradiation of samples was started after culture medium in petri-dishes was drawn out and the mouths of perti-dishes were sealed using 4μm sterilized mylar films.

Treatment after irradiation

As soon as irradiation ended, sealed films were taken out from petri-dishes, 2mL fresh 1640 culture

medium was then added into each petri-dish, petri-dishes with cells were then placed in CO_2 incubator and kept on cultivating. Twenty-four, 48, 72, 96 and 120h after cell culture, cells in 2 or 3 petri-dishes from each time point were treated by trypsinzation and stained with typan blue to identify the number of dead and living cells. Medium 1640 in other 2 or 3 petri-dishes from each time point was removed, cells were fixed for 4 hours with Carnoy's fluid, and stained for 10 minutes with acridine orange (0.01%, pH 6.8), rinsed three times with PBS buffer at pH 6.8, for 30min each time. Micronuclei were observed under fluorescence microscope. Materials in which micronuclei had been observed were rinsed with PBS and stained for 8min with Giemsa (1:20, pH6.8). Each petri-dish was observed under light microscope. Cells in 9 mesh eye piece micro-ruler were counted, and converted into cell numbers per area.

RESULTS AND DISCUSSION

The dependence of micronucleus frequencies on culture time for SMMC-7721

From Figure 1, it can be seen that micronucleus frequencies induced by single radiation of 0.68Gy and 6.8Gy and thrice radiation of 68Gy showed a tend ency to rise between 24h and 96h culture after radiation. The reas ons for this phenomenon might be that: ① micronuclei are delayed to appear following mitoses, as proposed by Mitchell and Norman[5]; ② mitoses take place later in cells with severe chromosomal damage expressed as micronuclei[6]. As compared with single radiation of 6.8Gy and thrice radiation of 68Gy, the micronucleus frequency of single radiation of 0.68Gy increased over 48h culture after slight irradiation (the micronucleus frequencies at 48h and 96h were 12.86% and 13.45%, respectively). This is because that cells irradiated with single dose of 0.68Gy are damaged to a less degree and easy to recover to normal through 48h culture, thereby the damaged cells inducing micronuclei became so low in number even reaching the number of normal cells. At about 96h, the micronucleus frequencies of cells irradiated with sin gle of 6.8Gy and thrice of 68Gy tended to fall (Figure 1). The reasons might be that: in these cells with less damage, DNA is repaired fast and easily recovered to normal; after culture for a certain duration, normal cells without micronuclei reproduce faster than the damaged cells with micronuclei, ratio (micronucleus frequencies) between the two was lowered.

Micronucleus frequencies are lowest at 48h for single and twice radiation of 68Gy, because the severely damaged cells have not recovered mitoses and only the less-damaged or non-damaged cells have had mitoses. After 48h cultu re, the severely damaged cells started mitoses, and the micronucleus frequencies rose significantly (Figure 1).

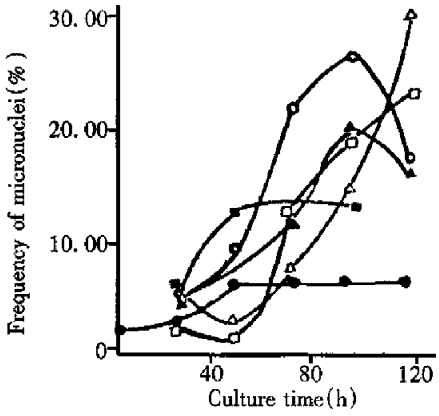

Figure 1 The dependence of micronucleus frequency on culture time for SMMC-7721.
(·) Control; (□) 0.68Gy; (·) 6.8Gy; (△) 68Gy; (□) Twice, 68Gy; (▲)Thrice, 68Gy

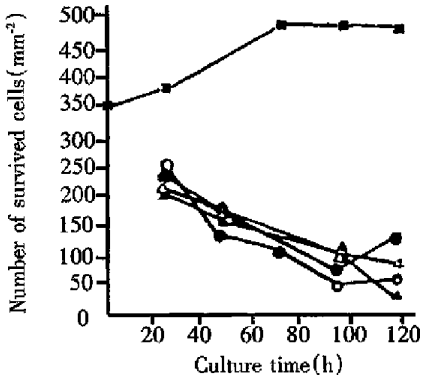

Figure 2 The dependence of number of living cells on culture time for SMMC-7721.
(□) Control; (·) 6.8Gy; (▲) 68Gy; (△) Twice, 68Gy; (·) Thrice, 68Gy

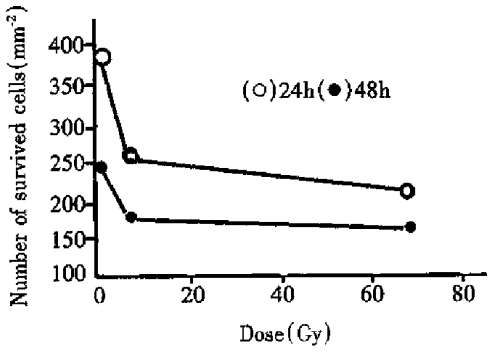

Figure 3 Dose response to living cells after differe nt culture time for SMMC-7721.

*Relationship between number of survived cells and culture time for SMMC-*7721

Figure 2 shows the relationship between the number of live cells and culture time for liver cancer cells. It can be seen that irradiated (single or fractionated)

liver cancer cells cultured for 24, 96 and 120 hours grew much slower than control. This showed that growth of liver cancer cells irradiated with heavy ions was obviously inhibited and some of liver cancer cells with different length of culture either exfoliated or died. As culture time was prolonged, survived control cells showed a rising tendency, whereas those of cells irradiated with heavy ions showed attenuating tendency. The latter resulted from radiation with high LET heavy ions which made the cell potentially lethal damage (PLD) enhance obviously. This appearance was also found in typan blue staining. In harvested cell suspension, some of the suspension cells were not deeply stained but stayed in anabiotic state which was a large proportion in irradiated materials. Comparison of single radiation of 6.8Gy, single, twice, and thrice of 68Gy found that the number of survived cells for single and twice radiation of 68Gy lowered gradually during the whole culture period. The difference is that after 96h culture, the number of survived cells for single radiation of 68 Gy decayed faster than those for twice radiation, as there was no interval in the single radiation of 68Gy, i.e., without chance of cell repair, and the number of induced reproductive dead cells increased more obviously than those for twice radiation. The number of survived cells with single radiation of 6.8Gy and thrice radiation of 68Gy increased after 96h culture, which indicates that cell damage by these two kinds of irradiation is slight, with lots of subletha l damage (SLD) cells. These SLD cells reactivated through certain time of cultur e, therefore, the survived cells increased after 96h culture.

Dynamic analysis of relationship between number of survived cells and micronucleus frequencies

The results in Tables 1 and 2 illustrate that micronucleus frequencies and survived cells increase or decrease with culture time with single and twice radiation of 68Gy (relative coefficient $r = -0.97$ and $r = -0.99$, respective ly). Micronucleus frequencies were negatively related to the number of survived cells. For single dose of 6.8Gy and 3 fraction doses of 68Gy, the micronucleus frequencies both reduced while the number of survived cells increased 96 hours a fter culture. It is indicated again that there was a negative relationship between micronucleus frequencies and the number of survived cells. Therefore micronucleus frequency is an important parameter describing the degree of the damaged cells.

Dose response to cell micronucleus frequencies after different culture time

Micronucleus frequencies of cancer cells after 96h culture were significantly higher than those of 24h culture with single radiation of 6.8Gy, single, twice and thrice radiation of 68Gy. Some researchers obtained a similar rule when studying irradiated lymphocytes[5]. The reasons are that induced micronucleus cells depend on mitosis while mitotic delay of the damaged cells (chromosome lesion) results in delayed production of large amounts of micronuclei. The changing rule of 24h culture was in agreement with the results by Shib amoto in his studies on lymphocytes[6]. After 96h culture, micronucleus frequencies of cells irradiated at a dose of 68Gy fell. This may be because that this kind of cancer cells were damaged more severely and inhibited cell mitotic procedure (mitosis is a prerequisite for micronuclei), therefore damaged cancer cells died before micronuclei were expressed. As compared with cells irradiated with 6.8Gy following 96h culture the micronucleus frequencies of cells irradiated with 68Gy decreased significantly.

Table 1 Relationship between micronuclei and cell survival in irradiated liver cancer with single irradiation

Cultural time (h)	Dose (Gy)	No. of scored cells	Frequency of micronuclei (%)	No. of Survived cells (mm^{-2})
24	6.8	2008	4.83	235
48	6.8	2047	9.19	175
72	6.8	2150	21.72	
96	6.8	1924	26.55	79
120	6.8	2096	17.45	129
24	68	1960	5.62	207
48	68	2150	3.00	158
72	68	2070	7.93	
96	68	1975	14.69	107
120	68	2040	30.39	36

Table 2 Relationship between micronuclei and cell survival in irradi ated liver cancer with fractionated irradiation

Cultural time (h)	Dose (Gy)	Time of irradiation	No. of scored cells	Frequency of micronuclei (%)	No. of Survived cells (mm^{-2})
24	68	2	971	5.13	213
48	68	2	1024	1.56	
72	68	2	964	12.79	
96	68	2	1152	19.01	101
120	68	2	1060	23.02	90
24	68	3	974	2.47	250
48	68	3	1050		136
72	68	3	1000	12.00	109
96	68	3	1000	20.00	50
120	68	3	1116	16.13	59

Dose response to survived cells after different culture time

Figure 3 shows the dose response to cell survival in human liver cancer cells. It can be seen that survival of liver cancer cells following 24h and 96h culture decreased with increase of radiation dose, i.e. cell

survival was negatively related to doses (r_{24h} = -0.68, r_{48h} = -0.70). When radiation dose was the same, the number of survived cells in human liver cancer cells for 48h culture was lower than that for 24h culture. The results showed that the number of survived cells of the irradiated liver cancer cells decreased gradually with culture time (survived number of control cells in creased with culture time), which results from reproductive death of lots of potentially lethal cells in irradiated cancer cells through several division cycles.

REFERENCES

1 Kraft G. Heavy ion therapy at GSI. *GSI Nachrichten*, 1993;11:3-5
2 Castro JR. Future research strategy for heavy ion radiotherapy. *Progress Radiooncol*, 1995;5:643-648
3 Linstadt DE, Castro JR, Phillips TL. Neon ion radiotherapy: results of the phase I/II clinical trial. *Int J Radiat Oncol Biol Phys*, 1991; 20:761-769
4 Sisterson J. Particles. *News Letter*, 1998;21:1-11
5 Mitchell JC, Norman A. The induction of micronuclei in human lymphocytes by low doses of radiation. *Int J Radiat Biol*, 1987; 52:527-535
6 Shibamoto Y, Streffer C, Fuhrmann C. Tumor radiosensitivity prediction by the cytokinesis block micronucleus assay. *Rediat Res*, 1991;128:293-300

Edited by MA Jing-Yun