Original Article
Effect of heavy ions and x-rays on proliferation and migration of malignant melanoma and its mechanism

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Abstract: Objective: This study aimed to investigate the effects of 12C6+ heavy ions (hereinafter referred to as heavy ions) with high linear energy transfer (LET) and x-ray irradiation with low LET on the proliferation and migration of melanoma in tumor-bearing mice, and to explore the mechanism. Methods: C57BL/6J mice were loaded with melanoma by implanting B16F10 cells, and were randomly divided into three groups. Mice in the two experimental groups were irradiated by the same physical dose of heavy ions and x-ray, respectively. Thirty days after irradiation, these mice were sacrificed at the same time. Proliferation-related and migration-related protein expression was detected by immunohistochemistry. After in vitro culture, B16F10 cells were irradiated with the same physical dose, and were collected 48 hours after irradiation. The expression of genes related to proliferation and migration was then detected by real-time polymerase chain reaction. Results: After tumor-bearing mice were irradiated, expression of the apoptosis inhibitory factor (survivin) and proliferation-related proteins (PCNA and Ki-67) were downregulated, and MMP-2/TIMP2 and MMP-9/TIMP-1 ratios decreased. These results indicated that radiation could promote apoptosis and inhibit proliferation of malignant melanoma cells, thereby inhibiting invasion and metastasis. Compared to x-rays, heavy ions have more pronounced promoting and/or inhibiting effects. Conclusion: Compared to x-rays, heavy ions have particular advantages in the treatment of malignant melanoma.

Keywords: Melanoma, heavy ions, X-ray, proliferation, migration

Introduction

Melanoma is a highly malignant tumor induced by genetic and environmental factors, which occurs mostly in the skin. The most important exogenous pathogenic factor is ultraviolet radiation from sunlight [1]. Malignant melanoma is generally caused by abnormal lemmocyte [2]. Lemmocyte can produce melanocytes. When the skin is stimulated by ultraviolet light, these cells may develop metabolic disorders, which usually manifest as disorders of pigment formation and tyrosine metabolism, and eventually lead to cancer. It is a highly aggressive malignant tumor with the highest mortality in skin tumors. The incidence of malignant melanoma continues to increase. Although this increase is partly due to increased awareness and improved screening techniques, it cannot be denied that malignant melanoma is becoming more prevalent [3].

After routine radiation exposure, the shoulders of the survival curves of malignant melanoma cells become widened, presenting a strong repair ability of sublethal damage. This suggests that melanoma cells are relatively insensitive to conventional radiation, and a malignant tumor is resistant to conventional radiation [4]. Compared to conventional radiation such as x-ray, heavy ion beams with high linear energy transfer (LET) have a Bragg peak at the end of the range, which allows sufficient amounts of radiation to be deposited in tumor tissues at the lowest possible dose in normal tissue. Furthermore, heavy ions have high relative biological effectiveness (RBE), low oxygen enhancement ratios (OERs), and confer tumor sensitivity, which are independent of the cell cycle. Therefore, heavy ion radiotherapy is considered to be an optimal radiotherapy mode [5-8].
In the present study, the inhibitory effects of heavy ions and x-rays on melanoma were studied at the cellular and animal levels.

Materials and methods

Cell line

Melanoma cells (B16F10) were purchased from Obio Technology (Shanghai) Corp., Ltd.

Animals

A total of sixty C57BL/6J pure line mice were purchased from the Experimental Animal Center of the Fourth Military Medical University, China. Half of the mice were male and half of the mice were female, and the average weight was 17±2 g (Animal qualification certificate no.: SCXX-[Army] 2012-0007).

Experimental consumables

Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from Biological Industries (USA), trypsin, 100 U/mL of penicillin and 100 μg/mL of streptomycin were purchased from Hyclone (USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma (USA), paraformaldehyde, phosphate buffered saline (PBS), anti-mouse monoclonal antibodies and S-P kits were used. Various models of flasks and Petri dishes were purchased from Corning (USA). The other reagents used were purchased locally at analytically pure levels.

Irradiation conditions

Heavy ion beams (\(^{12}\text{C}^+\) ion beams) were provided by the Heavy Ion Research Facility in Lanzhou (HIRFL) of the Institute of Modern Physics of the Chinese Academy of Sciences. The energy was 300 MeV/u, LET was 75 keV/μm, and dose rate was 0.15 Gy/min. The cells or mice to be irradiated were required to be perpendicularly placed in the Bragg peak area. X-ray beams with low LET were provided by the 21EX two-photon high-energy linear accelerator at the Lanzhou General Hospital of Lanzhou Military Area Command, with a dose rate of 4 Gy/min.

Tumor bearing

At the logarithmic phase, B16F10 melanoma cells were digested with trypsin, and the concentration of these cells was adjusted to 2 × 10\(^7\)/mL. Cells were then inoculated at the foot pads of these C57BL/6 mice at a dose of 0.1 mL per mouse. The presence of any exudation, swelling, and rupture in the foot pad of the mice was closely observed and timely recorded, as the mice were closely observed as a whole.

Subcutaneous solid tumors formed at the foot pads of the mice (approximately at the 11th day after tumor inoculation), and all mice presented with a mass at the planta (approximately 2.5 × 2.5 mm in diameter). The tumor bearing rate was 100%. These mice were randomly divided into three groups: control group, \(^{12}\text{C}^+\) (heavy ion) group and x-ray group (n=20, half were male and other half were female, each group).

Irradiation

A self-made device was used to fix the foot pads of the mice and facilitate the irradiation. The mice in both the heavy ion and x-ray groups were given a total dose of 10 Gy at the tumor site of the foot pad, while mice in the control group were not irradiated. All other conditions were the same.

Tumor management

Tumor-bearing mice were sacrificed by dislocation at 30 days after irradiation. Mice were weighed, and the tumor volume (length × width × thickness) was measured. The tumors were then carefully removed from the foot pads with ophthalmic scissors, photographed on a graduated paper and numbered. After that, the tumors were placed in the corresponding containers with 4% paraformaldehyde for storage.

Immunohistochemistry

Sections of the tumors were routinely dewaxed, and incubated with 0.3% methanol-hydrogen peroxide at room temperature for 20 minutes to eliminate endogenous peroxidase activity. The sections were washed three times with PBS for ten minutes each, underwent microwave antigen retrieval, the medium was diluted by the target antibody, and was left standing at 4°C overnight. Next, the secondary antibody was added and incubated for 30 minutes at 37°C, added with diaminobenzidine (DAB) color developing solution, re-stained with hematoxylin, and sealed. The sections were observed.
under a microscope. The first antibody was then replaced with PBS, and the other steps remaining unchanged. The above procedures were repeated once, and the results were used as negative control.

Detection of the expression of related protein genes by real-time polymerase chain reaction (PCR)

B16F10 cells were irradiated with heavy ions and x-ray for 48 hours, rinsed immaculately with PBS, added with 500 μl of Trizol for lysis on ice, and total RNA was routinely extracted. RNA concentration was calculated, and integrity was detected by 2% agarose electrophoresis. The total RNA concentration was adjusted to 50 mg/L. A 20-μl reaction system was prepared using the Prime Script RT reagent kit (TaKaRa), and reverse transcription was performed. A 20-μl polymerase chain reaction (PCR) reaction system was prepared using the SYBR Premix Ex Taq™ Real-time PCR kit, and real-time PCR testing of the RNA was performed according to kit instructions. The precautions were referred to the methods provided by Wong et al. [9]. Data were recorded, and the experimental results were statistically analyzed.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and were statistically processed using statistical software SPSS 22.0. In the univariate analysis, the numerical variables were evaluated by t-test, discrete variables were evaluated by X²-test or Fisher’s exact test, and Logistic multivariate regression analysis was performed on statistically significant indexes. P<0.05 was considered statistically significant.

Results

Effects of low LET rays and high LET particles on the expression of Ki-67 in melanoma in tumor-bearing mice

Ki-67 is a nuclear protein related to ribosomal RNA transcription, which can be used as a marker for cell proliferation. As shown in Figure 1, in the tumor tissues of tumor-bearing mice, the expression level of Ki-67 significantly decreased under the action of heavy ion and x-ray. These results revealed that the proliferation of tumor cells was retarded after radiation. Moreover, the expression level of Ki-67 in the heavy ion group was the lowest.

Effects of low LET rays and high LET particles on the expression of MMP-2 and MMP-9 in melanoma in tumor-bearing mice

The expression of MMPs was positively correlated with the malignant degree of melanoma [10, 11]. As shown in Figures 2 and 3, the over-
expression level could reflect the high invasive biological characteristics of melanoma. This experiment revealed that expression of MMP-2 and MMP-9 in melanoma was significantly strongly positive in the control group. Furthermore, it could be observed that the positive rate significantly decreased after irradiation, the positive rate of melanoma after high LET radiation was lower than that after x-ray irradiation, and there were very few positive cells in the visual field. This indicates that metastasis and angiogenesis of tumor cells were inhibited by heavy ion irradiation.

Effects of low LET rays and high LET particles on the expression of TIMP-1 and TIMP-2 in melanoma in tumor-bearing mice

Tissue inhibitors of metalloproteinase (TIMPs) are the natural inhibitors of matrix metalloproteinases (MMPs). As shown in Figure 4, TIMP-1 was expressed in the three groups. Its expression level increased in the irradiation groups, and part of the cells presented a strongly positive expression. As shown in Figure 5, the expression of TIMP-2 in tumor tissue increased to a certain extent after irradiation. These results suggest that expression of TIMP-2 was increased after tumors were irradiated, its inhibitory effect on MMPs was enhanced, and cell migration was inhibited through this pathway.

Effects of heavy ions and x-ray on the expression of different genes in B16F10 cells

The expression of apoptosis-related and proliferation-related genes in cells after radiation was detected by real-time PCR. As shown in Figure 6, after cells were irradiated and the expression levels of proliferation-related genes (Ki-67, PCNA, and Survivin) decreased. Compared with the control group,
the expression level of Ki-67 decreased by 27.09±0.152 times, the expression level of PCNA decreased by 6.46±0.503 times, and the expression level of survivin decreased by 2.84±0.114 times; and the differences were statistically significant (P=0.006, P=0.046, and P=0.009). As shown in Figure 7, after B16F10 cells were irradiated, the expression of invasion-related and metastasis-related genes (MMP-2, MMP-9, TIMP-1, and TIMP-2) all changed. Real-time fluorescent quantitative PCR revealed that cell irradiation could downregulate expression of MMP-2 and MMP-9 genes, compared with the cells in the control group. In the heavy ion group, the expression level of MMP-2 decreased by 10.07±0.251 times, and the expression level of MMP-9 decreased by 4.31±0.216 times. Meanwhile, the expression of TIMP-1 and TIMP-2 genes was upregulated. The expression level of TIMP-1 increased by 17.6±0.335 times and the expression level of TIMP-2 increased by 2.19±0.206 times and the differences were statistically significant (P=0.018, P=0.034, P=0.014, and P=0.020). Furthermore, the MMP-2/TIMP2 and MMP-9/TIMP-1 ratios also decreased. These results reveal that irradiation, especially heavy ion irradiation, can inhibit the proliferation, infiltration and metastasis of malignant melanoma cells in mice.

**Discussion**

Under normal physiological conditions, proliferation and apoptosis of cells maintain homeostasis, and play a key role in maintaining homeostasis and resisting the interference of external factors. Excessive cell proliferation or apoptosis inhibition would lead to the occurrence and development of tumors. Cell proliferation is a basic feature of cellular life, and is closely related to the growth, physiological regeneration, wound repair, programmed cell death, and tumor development of an organism. The proliferative capacity of a tumor is closely related to its biological characteristics, clinical manifestations and treatment response in the later stage. Cell proliferation markers can accurately and objectively reflect its proliferative potential [13].

Ki-67 is a ubiquitous symbolic antigen in human proliferating nuclei. It is expressed in the proliferating stage of cells, and is closely related to the cell cycle [14, 15]. It is one of the most widely used proliferating cell markers, and its expression in cells is strictly regulated. Furthermore, it can indirectly represent the proliferative activity of tumor cells [16]. Its expression level is usually positively correlated with the extent of cell proliferation, and is also highly correlated to the development, metastasis and
Migration of malignant melanoma by different rays

The proliferation and apoptosis in normal cells to ensure the normal growth and development of the body. This process requires close coordination and unified regulation of cell proliferation, differentiation and apoptosis. Proliferating cell nuclear antigen (PCNA) is a nuclear protein necessary for DNA synthesis in eukaryotic cells, is a marker for the expression of DNA polyploid forms, and serves as a marker of tumor cells under dysregulation. The proliferation and cancer-forming extent of malignant tumors can be estimated by detecting Ki-67 and PCNA. This has clinical significance in assisting diagnosis, estimating prognosis, and guiding treatment. Furthermore, the present study also revealed that expression levels of Ki-67 and PCNA significantly decreased under the action of heavy ions and x-rays. These results suggest that proliferation of cells retarded after radiation, and this retarding effect was more significant in the heavy ion group. In another article [19], it was also revealed that heavy ion irradiation could reduce expression level of apoptosis inhibitory factor survivin in malignant melanoma, activate apoptosis-related factors, and promote the apoptosis of melanoma cells. The proliferation indicators in these cells were significantly downregulated after heavy ion irradiation. These results indicate that heavy ion radiotherapy retards the proliferation of melanoma in mice, induces apoptosis, and inhibits the occurrence and development of malignant melanoma by interfering with the balance between proliferation and apoptosis.

Metastasis is the biggest threat to the survival and quality of life of cancer patients. The ultimate goal of cancer treatment is to remove the primary tumors and any potential metastases. It is an important direction of oncology research to deeply understand the mechanism and regularity of the invasion and metastasis of malignant tumors. In the process of infiltration and metastasis, the extracellular matrix is the main obstacle for the abscission and migration of tumor cells. In the process of the evolution of tumors from in situ proliferation to infiltration and metastasis, the extracellular matrix and basement membrane must be degraded and MMPs and its endogenous inhibitors (TIMPs) play important roles in this process [20-22]. MMPs can promote the metastasis of tumor cells by degrading the extracellular matrix of these tumor cells, while TIMPs can be directly inhibited by corresponding endogenous inhibitors [23, 24]. Bafetti and Hiro-omi, et al. [25] revealed in their study that tumor cells in abnormal differentiation can induce surrounding interstitial cells to secrete MMP-9 and destroy the normal surrounding microenvironment to increase their infiltration and metastasis ability. Studies on various types of tumors in humans have revealed that enhancement of the expression of MMP-9 was closely correlated to the invasion and metastasis of tumors, and the expression level of MMP-2 was positively correlated with the malignant degree of tumor cells [26, 27], and closely correlated to the local invasion and metastasis of malignant melanoma. NJ Song et al. [28] revealed that expression of MMP-2 was positively correlated with the expression of MMP-9 in malignant melanoma. This was consistent with the results in this experiment. Expression of MMP-9 in malignant melanoma can promote the invasion and metastasis of tumors [29]. This experiment revealed that expression levels of MMP-2 and MMP-9 significantly decreased in the irradiated groups, compared with the control group. Furthermore, the expression level of MMP-2 in tumor-bearing mice decreased to a certain extent after local x-ray irradiation on the tumor, but a strong positive expression could still be observed in some cells. These results reveal that x-ray has a certain inhibitory effect on the infiltration and metastasis of malignant melanoma, while the inhibitory effect in the heavy ion group was more significant. Furthermore, it could be observed that the positive expression level was significantly lower in the visual field, compared with the control group. This suggests that heavy ions with high LET has inhibitory effects on the infiltration and metastasis of cells insensitive to x-ray irradiation due to its biological properties.

TIMP is a natural inhibitor of MMPs. There is a certain degree of specificity between MMPs and TIMPs. TIMP-1 can inhibit most MMPs, but mainly selectively inhibits MMP-9; and TIMP-2 mainly specifically inhibits MMP-2 [30, 31]. The present study revealed that the MMP-2/TIMP2 and MMP-9/TIMP-1 ratios increased with the increase in the malignant degree of tumors. Furthermore, the secretion of TIMPs relative to
Migration of malignant melanoma by different rays

MMPs was seriously insufficient, and the action of MMPs significantly increased, allowing the tumor to be highly invasive. A balance exists between MMP-9 and TIMP-1 in normal tissues, which maintains the stability of the extracellular matrix. Expression of MMPs and TIMPs was upregulated during carcinogenesis, but the extent of this increase in TIMPs was not enough to inhibit the activity of MMPs. Hence, the balance between these two was broken, which led to the excessive degradation of ECM. TIMP-1 blocks the activation of enzymes by respectively combining with the zymogen form of MMP-2 and MMP-9 at a ratio of 1:1, and accordingly plays a role in inhibition of tumor growth and prevention of tumor invasion and metastasis. Therefore, the TIMPs/MMPs dynamic balance is more significant for evaluating invasion and metastasis of melanoma under radiation. This experiment reveals that expression levels of TIMP-1 are upregulated in tumor cells and the expression level of TIMP-2 is not significantly changed after irradiation, and the difference between the heavy ion group and x-ray group is not statistically significant. A study also revealed that TIMP-2 RNA and TIMP-2 protein can also be expressed in malignant melanoma cells, and even increase in expression level, when compared to normal tissues. However, TIMP-2 in cells slightly changed after irradiation and the expression level of MMP-2 was downregulated, and the MMP-2/TIMP-9 ratio decreased. These results reveal that the invasion and metastasis ability of irradiated cells is decreased. In particular, after heavy ion irradiation, the decrease in this ratio was more pronounced. This suggests that the MMP/TIMP ratio was downregulated in tumor tissues after radiation. Thus, the activation of MMP zymogen could be suppressed, and the effect of high LET beams is more pronounced, inhibiting the invasive ability of malignant melanoma. The above results are similar to those reported by Yang Liu et al. [32] High LET carbon ion radiation can better inhibit migration of glioma and endothelial cells compared to low LET X-rays, and inhibit tumor growth and angiogenesis by regulating vascular endothelial growth factor (VEGF)-mediated signaling pathway in the tumor microenvironment. Kazutoshi Murata also mentioned that [33] carbon ion irradiation can effectively inhibit metastatic potential of A549 cells by inhibiting the PI3K/Akt signaling pathway. Furthermore, Iris Eke’s [34] study also revealed that carbon ion radiation could effectively reduce the invasion ability of glioma.

The occurrence of tumors is a complicated process related to multiple genes, multiple steps and multiple variates. Malignant melanoma is even more complicated. In recent years, with the continuous development of molecular biology, the molecular mechanisms of radiotherapy have also been explored. The present study revealed that the expression of survivin, PCNA, and Ki-67 was downregulated, and MMP-2/TIMP2 and MMP-9/TIMP-1 ratios decreased after heavy ion irradiation. Compared to x-rays, heavy ion radiation could promote the apoptosis of malignant melanoma cells and inhibit its proliferation, and more obviously break the imbalance between the proliferation and apoptosis of tumor cells, and promote its apoptosis and inhibit its proliferation. It is hoped that the low radiation sensitivity of malignant melanoma can be overcome. For invasion and metastasis, heavy ion irradiation can more effectively inhibit the metastatic potential of malignant melanoma. The present study provides a theoretical basis for the clinical trials of heavy ion therapy for malignant melanoma and helps to accurately determine a heavy ion treatment regimen.

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Migration of malignant melanoma by different rays

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