Protective Effects of Melatonin Against Low- and High-LET Irradiation

Guangming ZHOU¹, Tetsuya KAWATA³, Yoshiya FURUSAWA²*, Mizuho AOKI², Ryoichi HIRAYAMA², Koichi ANDO² and Hisao ITO³

Melatonin/Antioxidant/Mammalian cells/X-rays/Carbon beam.

To investigate the protective effects of melatonin against high-LET ionizing radiation, V79 Chinese hamster cells were irradiated with 100 keV/µm carbon beam. Parallel experiments were performed with 200 kV X-rays. To avoid the impact from extra solvents, melatonin was dissolved directly in culture medium. Cells were cultured in melatonin medium for 1 hr before irradiation. Cell inactivation was measured with conventional colony forming assay, medium containing 6-thioguanine was used for the selection of mutants at hprt locus, and the cell cycle was monitored by flow cytometry. Both carbon beam and X-rays induced cell inactivation, hprt gene mutation and cell cycle G2 block dose-dependently. But carbon beam showed stronger effects as indicated by all three endpoints and the relative biological effectiveness (RBE) was 3.5 for cell killing (at 10% survival level) and 2.9 for mutation induction (at $5 \times 10^{-5}$ mutants/cell level). Melatonin showed protective effects against ionizing radiation in a dose-dependent manner. In terms of cell killing, melatonin only increased the survival level of those samples exposed to 8Gy or larger of X-rays or 6 Gy or larger of carbon beam. In the induction of hprt mutation and G2 block, melatonin reduced such effects induced by carbon beam but not by X-rays. The results suggest that melatonin reduces the direct interaction of particles with cells rather than an indirect interaction. Further studies are required to disclose the underlying mechanisms.

INTRODUCTION

Melatonin, N-acetyl-5-methoxy tryptamine, is a secretory product of the pineal gland and well known for its functional versatility.¹² It has been verified as a direct free radical scavenger and an indirect antioxidant at both physiological and pharmacological concentrations.³⁴ The radioprotective effects of melatonin against UVB⁵ and near infrared radiation⁶ have been reported.

Ionizing radiation is classified as a potent carcinogen.⁷ Exposure of cells to ionizing radiation results in immediate and widespread oxidative damages to DNA by both direct and indirect mechanisms. Direct action produces disruption of chemical bonds in the molecular structure of DNA while indirect effects result from highly reactive free radicals such as ·OH, ·H and eaq produced during the radiolysis of water, and their subsequent interaction with cellular DNA. Therefore, melatonin has been expected to have certain radioprotective effects against ionizing radiation. Koc et al.⁸ found that melatonin administration prior to irradiation prevented γ-ray-radiation induced damage on rat peripheral blood cells in vivo. Karšlioğlu et al.⁹ reported that melatonin supplementation increased the activity of superoxide dismutase and glutathione peroxidase and subsequently decreased the lipid peroxidation level, protecting rats from γ-ray-radiation induced cataract formation. Vijayalaxmi et al.¹⁰ proved that melatonin also protected human peripheral blood cells against γ-rays in vitro. The protective effects of melatonin against X-rays were also verified both in vivo and in vitro.¹¹ The antioxidant effects of melatonin against low-LET irradiation were also reported by Sener et al.¹²,¹³ Energized heavy ions belong to high-LET radiation, physically different from low-LET particles like X-ray and γ-rays. But high-LET irradiation also induces large amount of free radicals while interacting with materials like low-LET irradiation. Melatonin can be expected to reduce high-LET-radiation induced biological effects by its free radical scavenging ability. To the best of our knowledge, the present article is the
first to elucidate the radioprotective effects of melatonin against irradiation with carbon beam.

Another purpose of our study was to test the protective effects of melatonin in a condition without any impact from extra solvents. According to published reports, ethanol is a very commonly used solvent for dissolving melatonin. While performing experiments, investigators usually set up a control sample receiving the same volume of ethanol and the same amount of irradiation, and little influence of ethanol was reported. However, evidence has been presented to show that ethanol reduces the DNA-damaging effects of two important oxidants - hydrogen peroxide and ionizing radiation. Monobe and Ando found that the addition of 10 mM ethanol to blood samples before X-ray irradiation reduced chromosome aberrations. The number of dicentrics per cell decreased from 1.75 to 1.30, and the number of acentric fragments per cell decreased from 2.91 to 2.34. In order to avoid the solvent issue and observe the actual radioprotective effects of melatonin, we dissolved melatonin directly in culture medium. The concentration used in this study was 0.43 mM, much higher than physiological concentration. This means that the difference between melatonin-treated samples and non-melatonin-treated samples in this study can be reliably ascribed to the effect of melatonin.

MATERIALS AND METHODS

Cell culture

Chinese hamster lung fibroblast cells V79 were used throughout the experiments. Briefly, cells were grown as monolayers in F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS, Lenexa), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C, in 5% CO2 and 95–100% relative humidity.

Exponentially growing cells were harvested and re-seeded in T25 culture flasks one day before irradiation at a density of 2 × 10^5 cells/flask.

Melatonin treatment

Melatonin was purchased from Sigma-Aldrich. According to the specifications, the maximum solubility was 0.1 mg/ml, and thus 10 mg of melatonin was directly dissolved in 100 ml of F12 medium by thorough stirring for about 1 h in the dark and the solution was then filtered for sterilization to make up 0.43 mM solution. The solution was freshly made right before each set of experiments. One hour before irradiation, the medium in each flask was replaced with 5 ml of melatonin solution and the flasks were kept in a CO2 incubator until irradiation. Media of control samples were also replaced with fresh F12 medium.

Irradiation

Carbon beams with initial energy of 290 MeV/u were generated by HIMAC (Heavy Ion Medical Accelerator in Chiba) at the National Institute of Radiological Sciences, Japan. Modulator films were used to perform a LET value of 100 keV/µm. Samples were irradiated at room temperature. Control samples were treated by the same protocol except for irradiation.

A Pantak-320S X-ray facility (Shimadzu, Tokyo) was operated with 0.5 mm Al and 0.5 mm Cu filters at 200 kV and 20 mA. The dose rate was 1 Gy/min. All irradiations were carried out at room temperature.

Colony forming assay

Cell survival was measured by conventional colony forming assay. Briefly, cells were collected by trypsinization, resuspended in F12 medium supplemented with 10% FCS, diluted and seeded in 60-mm Petri dishes to provide 50–100 colonies per dish. Dishes were incubated for 6 days, then fixed with 10% formalin in PBS and stained with 1% methylene blue. Colonies containing more than 50 cells were identified as survivors under a stereomicroscope. A modified single-hit multitarget model was employed to analyze the survival data. The formula used was

\[ S = z e^{-D/D_0} \left[ 1 - (1 - e^{-D/D_0})^n \right], \]

where the inactivation coefficients \( D_1 \) and \( D_2 \) of the single-hit term and the multitarget term, respectively, are related to the final slope or the mean lethal dose \( D_0 \) by the relationship of \( D_0^{-1} = D_1^{-1} + D_2^{-1} \). The third parameter \( z \) allows the fitting at the origin to deviate from 100% survival for the best fit, and \( n \) is the extrapolation number.

hprt gene mutation

The method used to determine the frequency of radiation-induced hprt mutants has been described. Briefly, the cells were allowed a further 8 days to express the hprt mutants after irradiation. And then, 5 × 10^5 cells were seeded in selective medium containing 30 µM 6-thioguanine (6-TG, Sigma). At the same time, a separate set of dishes was plated in a medium lacking 6-TG to determine the cloning efficiency. Dishes were incubated for 6 days, then fixed with 10% formalin in PBS and stained with 1% methylene blue. Colonies containing more than 50 cells were identified as mutants under a stereomicroscope. This mutation frequency was corrected with the cloning efficiency of the cells at the time of selection. The induced frequencies were calculated by subtracting the background frequencies in the control population.

Cell Cycle Assay

Cells post-incubated for 24 hr were harvested and fixed with 70% pre-chilled ethanol for over 24 hr at -20°C, suspended in PBS, treated with 100 µg/ml RNase A (DNase free, Sigma), and stained with 50 µg/ml propidium iodide (Sigma) for 30 min at 37°C. Cell cycle distribution was analyzed with Modfit software (Verity Software, Topsham,
Maine, USA) from the histogram of DNA content measured with a flow cytometer (FACScan, Becton Dickinson, California, USA).

Statistical analysis

The parameters were calculated by the least-squares method of a computer program (KaleidaGraph, Synergy Software, Philadelphia, USA).

All the experiments were repeated at least three times, and the data were presented as mean ± standard error margin (SEM). Significance levels were assessed by Student’s t-test, and p < 0.05 was considered statistically significant.

RESULTS

Survival fractions of V79 cells are plotted in Fig. 1, and the parameters obtained with a modified single-hit multitarget model are listed in Table 1. Apparently, both X-ray and carbon beam resulted in a dose-dependent inactivation of V79 cells. The survival curve of X-rays showed a wide shoulder and the dose required for 10% survival ($D_{10}$) was 8.56 Gy, while that of carbon beam showed little shoulder and $D_{10}$ was 2.44 Gy, meaning that the relative biological effectiveness (RBE) of 100 keV/µm carbon beam was 3.51. Carbon irradiation clearly has higher inactivation ability than X-ray irradiation.

For X-ray irradiation, pretreatment with melatonin increases the survival fraction of high-dose irradiated cells. Significant difference in survival was induced by either 8 or 10 Gy of X-rays. When the dose was less than 6.8 Gy, there was no significant difference in the survival level between samples with and without melatonin treatment. Therefore, pretreatment with melatonin does not change the shoulder of the survival curve but rather the slope of the exponential portion in the survival curves. As shown in Table 1, $D_0$ describing the change in slope increased from 3.64 Gy to 4.68 Gy.

Similar results were obtained with carbon irradiation. Melatonin seemed to protect cells against carbon irradiation, but there was no significant difference between corresponding samples with or without melatonin treatment when the dose was less than 5 Gy. However, when induced with 6 Gy of carbon beam, the survival fraction of V79 cells pretreated with melatonin was significantly higher than that without melatonin treatment.

Figure 2 shows the $hprt$ gene mutation frequencies in V79 cells as a function of irradiation dose, and the data were fitted with a linear-quadratic formula. Both X-ray and carbon irradiations induced the mutation at the $hprt$ locus in a dose-dependent manner, but carbon beam showed higher ability in mutation induction than X-rays. The RBE at the induction of $5 \times 10^{-5}$ mutants per cells was 2.93.

Melatonin treatment showed no protective response against $hprt$ mutation when the X-ray dose was less than 5 Gy, but...
Gy. When doses exceeded 5 Gy, the mutation frequency of melatonin-treated cells seemed lower, resulting in a slight change in the slope of the mutation-induction curve. However, there was no significant difference between two samples exposed to the same dose although one was in melatonin medium during irradiation while the other was not. For the mutation induction with carbon beam, melatonin treatment changed the shape of the mutation-induction curve and melatonin-treated samples showed lower mutations than the parallel samples.

As the RBE values are of interest for radiation protection purposes, we calculated RBE based on the curves shown in Figs 1 and 2. The results are shown in Fig. 3. Due to the hypersensitivity and other de novo discoveries in the low-dose range less than 0.5 Gy, the curves were not extrapolated from 0.5 Gy to 0 Gy. As shown in Fig. 3, both RBE curves of cell killing and mutation induction decreased along with the doses. The ratio of these two RBEs varied slightly between 0.87 at 0.5 Gy and 1.0 at 10 Gy, meaning that mutagenicity of 100 keV/µm was no more than X-rays in this dose range. Melatonin markedly changed the RBE curves, especially flattening that of mutation induction much. The ratio of the two RBEs of mutation induction and cell killing gradually increased from 0.57 at 0.5 Gy to 1.13 at 10 Gy. These results imply that melatonin relatively reduces a relative reduction in the mutagenicity of carbon beam at a low-dose range rather than a high-dose range.

Both X-rays and carbon beam blocked V79 cells in the G2 phase. As shown in Fig. 4, the dose response in G2-block induction by X-rays was linear while that induced by carbon beam was linear-quadratic. Carbon beam induced G2-block much more efficiently than X-rays. In 24-hr post-irradiation with 7 Gy of carbon beam, cells in the G2/M phase...
increased 3.25 ± 0.21 folds.

The G2 block induced in melatonin-treated cells was correspondingly lower than that in cells without melatonin treatment, especially when the cells were exposed to carbon beam. For X-ray irradiation, less melatonin-pretreated cells seemed to be blocked in the G2 phase but the difference with untreated cells was not significant.

**DISCUSSION**

Tumor therapy with carbon beam is now clinically used in Japan and Germany because of its outstanding physical and biological characteristics. In the present study, all the data concerning cell killing, mutation induction and cell cycle change showed that carbon beam had much stronger effects than X-rays, which was consistent with former studies and confirmed the superiority of carbon beam in tumor therapy. Moreover, the present study was the first to demonstrate the protective ability of melatonin against high-LET-radiation induced damage, as represented by cell killing, hprt gene mutation and cell cycle block.

Melatonin scavenges •OH with a very high rate constant, roughly in the order of 2.7 × 10^{10} M^{-1}S^{-1}, and about 60–70% of cellular DNA damage produced by low-LET ionizing radiation is estimated to be caused by •OH, formed by the radiolysis of water. Therefore, melatonin could be expected to have protective effects against low-LET ionizing radiation, as has been widely verified both in vitro and in vivo. Kim et al. verified that melatonin pretreatment inhibited radiation-induced apoptosis with 8 Gy of X-rays by inhibition of lipid peroxidation. In the present study, we measured the dose response of 3 kinds of endpoints. To the best of our knowledge, this was the first to study the radioprotective effects of melatonin with such a wide radiation dose range.

Melatonin increases the survival fraction in a dose-dependent manner. In the low-dose range, melatonin shows no protective effect. Significant increments of survival level are seen in cells irradiated with 8 or 10 Gy of X-rays. The shoulder of survival curves is hardly altered by pretreatment with melatonin, but the slope of the exponential portion of the survival curves is greatly affected. The survival data of carbon beam are distinctly reduced by melatonin. 100 keV/mm Carbon beam belongs to densely ionizing radiation, which is more effective in causing biological damage because more of its energy is released in clusters of ionizing events, giving rise to more severe local damage. Direct action is the dominant process in the interaction of high-LET particles with biological materials. Melatonin shows no effects on the gene mutation and cell cycle block induced by X-rays but does on those induced by carbon beam, which implies that melatonin modifies the direct interaction of high-LET particles with biomolecules.

Since this is the first time for this hypothesis to be presented, namely that melatonin likely protects cells against ionizing radiation-induced damages by lessening the direct interaction of ionizing radiation with cells, the mechanism still needs to be unraveled. One of the premises of this hypothesis is that melatonin is in sufficient proximity to DNA molecules during irradiation. The studies on melatonin receptors seem to support this possibility. It has been elucidated that the receptor RZR/ROR is an important member of the nuclear receptor superfamily, and it has recently been shown to be the nuclear receptor for melatonin. RZR/ROR binds as a monomer to DNA, and the human 5-lipoxygenase gene has been identified as the first RZR/ROR/melatonin-responder gene. Thus, with melatonin binding to DNA molecules through the receptor RZR/ROR, the notion that melatonin lessens the direct reaction of ionizing radiation with DNA molecules becomes a distinct possibility. Further experiments are required to disclose the underlying mechanisms.

It is reported that protective effects of melatonin against low-LET radiations are due to its scavenging ability for hydroxyl radicals. In contrast, we found that melatonin shows protective effects against the direct action of ionizing radiation. As for the differences between our experiments and other reports, following factors can be considered. The first concerns the concentration of melatonin. We used the a saturated concentration, 0.43 mM, whereas Vijayalaxmi et al. used 0.5 mM through 2 mM while Kim et al. and Lee et al. used much lower concentrations of 10 μM and 0.1 μM, respectively. With such a wide concentration range, comparing the results is difficult. The second factor involves the solvent. Although ethanol is widely used for dissolving melatonin, its free radical scavenging ability may not be sufficiently taken into account. For this reason, we dissolved melatonin directly dissolved in cell culture medium to preclude any solvent-related uncertain consequence. Future parallel experiments with different solvents will be helpful for clarifying such uncertainty. The third factor is that of the cell line. The hprt gene mutation represents a very useful assay system for investigation of the mutagenic action of ionizing radiation, but the change in the exponential portion is due to the direct action of particles. This suggests that melatonin probably exerts a protective response against direct effects induced by ionizing radiation, instead of free radical scavenging activities as reported.

This hypothesis is emphasized by the data of mutation induction and cell cycle block. Melatonin gives no significant protective response against X-ray irradiation although it seems to reduce mutation induction and cell cycle block. However, the hprt gene mutation and cell cycle block induced by carbon beam are distinctly reduced by melatonin. 100 keV/mm Carbon beam belongs to densely ionizing radiation, which is more effective in causing biological damage because more of its energy is released in clusters of ionizing events, giving rise to more severe local damage. Direct action is the dominant process in the interaction of high-LET particles with biological materials. Melatonin shows no effects on the gene mutation and cell cycle block induced by X-rays but does on those induced by carbon beam, which implies that melatonin modifies the direct interaction of high-LET particles with biomolecules.
radiations, and V79 cell line used in this study is quite acceptable for hprt gene mutation studies.\textsuperscript{33} Unfortunately, this cell line is seldom used in the evaluation of radioprotective effects of melatonin. It has been widely found that melatonin modulates cell proliferation in cell-type-dependent manner,\textsuperscript{34,35} which implies that melatonin probably has various effects on different cell lines.

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