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ORIGINAL RESEARCH

Evaluation of the degree of medical radiation damage with a highly sensitive chemiluminescence method

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ABSTRACT: A highly sensitive chemiluminescence (CL) method for evaluation of medical radiation damage degree is presented. According to the principle of cell stress response to ionizing radiation, lymphocytes will produce reactive oxygen species (ROS) after irradiation. The ROS produced can react with 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-α] pyrazin-3-one (MCLA), a specific CL probe for superoxide anion ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$), to emit light at 465 nm. The CL intensity is positively related to the amount of generated ROS detected 30 min after irradiation. Cell viability, which is inversely related to cell mortality, was determined by MTT assay after 3 days' culture. The results show that both CL intensity and cell mortality of lymphocytes increase with the increase of the radiation dose when the dosage is no more than 3 Gy, suggesting a positive relationship between the degree of lymphocyte cell damage and the amount of ROS generated. In addition, the effects of catalase, Cu–Zn superoxide dismutase (SOD), mannitol, sodium azide (NaN_3), and D_2O on MCLA-dependent CL of lymphocytes are discussed. We believe that the MCLA-dependent CL method would potentially provide an easy way for evaluating the degree of lymphocyte damage induced by radiation. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: radiation damage degree; chemiluminescence; reactive oxygen species; MCLA; lymphocyte

INTRODUCTION

Radiation therapy is one of the most important methods in malignant tumour treatment, but it can also damage normal tissue. The biological effect of radiation is very complex. It can induce a tumour, lead to gene mutation, destroy the structure and inhibit the biosynthesis of nucleic acids and proteins. However, it can also cure a tumour, restore the mutant gene, accelerate the biosynthesis of nucleic acids and proteins, and accelerate cell division and organism growth. Hence, estimating the safety of radiation therapy is very important. The conventional evaluation of the degree of radiation damage mostly depends on lymphocyte viability tests using the 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) method (1–4) or cell counting results, or sometimes the lymphocytes micronucleus frequency test (5–7). All these methods are complex and time consuming, and cells often need to be cultured for a period of time before assay.

Chemiluminescence (CL), a luminescence resulting from a chemical reaction, has become a standard tool in biomedical research. It is widely used in immunoassay, cell detection, nucleic acid testing and drug discovery (8).

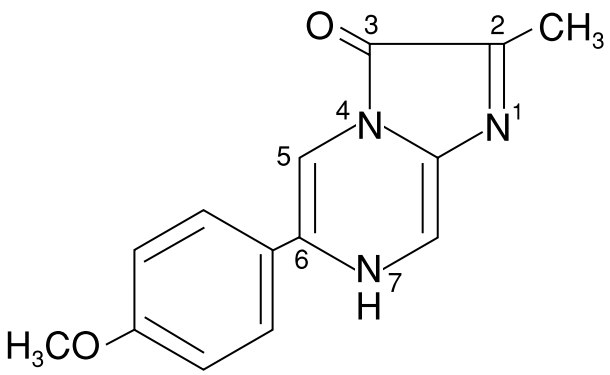

Figure 1. The chemical structure of MCLA.

2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-α] pyrazin-3-one (MCLA) is a specific and selective CL probe to detect superoxide anion ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) (9–12) (Fig. 1). The mechanism of reaction between MCLA with $^1O_2$ or $O_2^{\cdot-}$ probably involves a dioxetane analogue, which decarboxylates and protonates to an excited carbonyl compound, which de-excites to emit light at 465 nm (13).

In the present study, we developed an effective CL method to analyse lymphocytes damage caused by γ-irradiation. MCLA was used as a CL probe to determine the amount of irradiation-induced reactive oxygen species (ROS) generated by lymphocytes (14).

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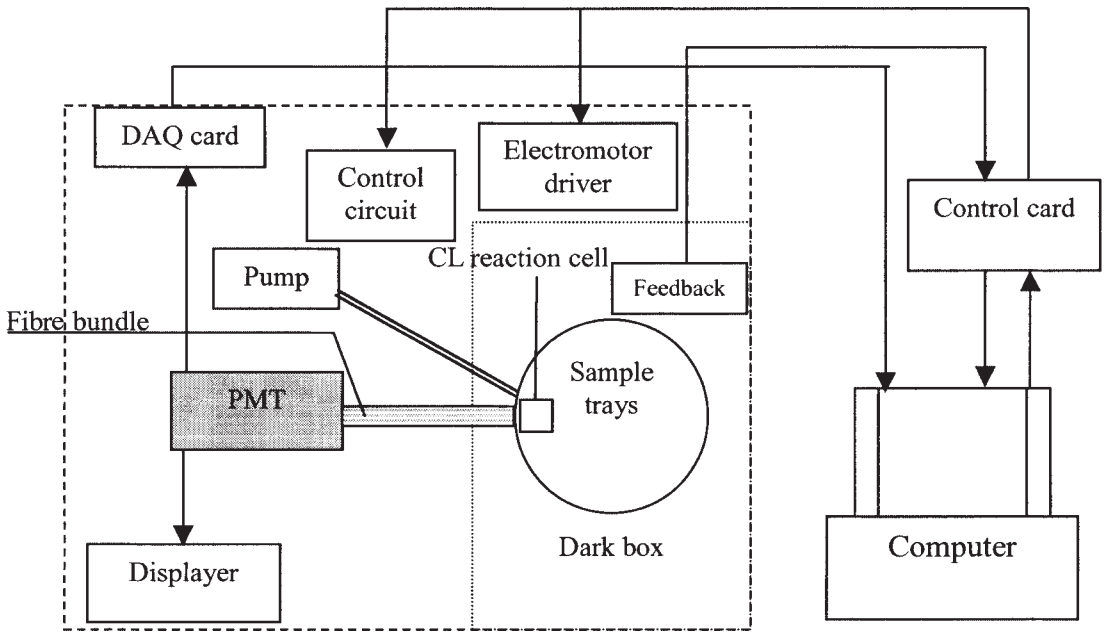

Figure 2. The diagram of biophoton cell function analyser.

MATERIALS AND METHODS

Samples and reagents

Anticoagulated peripheral blood (AB type, 100 ml) was obtained from Guangzhou Blood Bank. Lymphocyte separation medium was purchased from Tianjin Haoyang Biological Manufacture Co. Ltd. MCLA was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Sodium azide (NaN_3) is AR grade and made in China. Cu–Zn superoxide dismutase (SOD) was purchased from Sigma. MCLA, NaN_3 and SOD were performed by deionized double-distilled water just before use.

Experimental set-up

The experimental set-up, using a biophoton cell function analyser (Fig. 2) to record the CL signal, was established in our laboratory (15). CL reaction cells were made of 18 standard quartz cuvettes ($0.5 \times 1 \times 4$ cm). A stepper motor drove the reaction cells to move in turn. An optical fibre bundle received the light emitted during the CL reaction and transmitted it to an ultra-high-sensitivity single-photon counting module (PMT, MP-962; Perkin-Elmer, Wiesbaden, Gemany), which has a linear response range with six orders and can detect spectra of 200–850 nm. The signal from the PMT was amplified and discriminated. Transistor–transistor logic (TTL) pulses were counted every second with a multi-function acquisition card (PCL-836; Advantech, Taiwan) controlled by Labview software. The signal collection process and data analysis were accomplished with a personal computer.

Lymphocytes separation

Lymphocytes were isolated by density gradient centrifugation using the lymphocytes separation medium. Briefly, anticoagulated peripheral blood was overlayed on lymphocyte separation medium with the proportion of 1:2 and centrifuged at 1500 r/min and 20°C for 10 min. Then, the supernatant plasma was collected. The interface layer mainly containing lymphocytes was washed twice with RPMI 1640 medium at 2000 r/min and 20°C for 10 min. Cells were adjusted to 6×10^6 cells/mL in RPMI 1640 medium. The percentages of lymphocytes, monocytes and neutrophils were 85%, 13.5% and 1.5%, respectively, estimated using a Giemsa stain.

Irradiation

Whole blood samples and lymphocytes suspensions were divided into nine aliquots. The aliquots were exposed to doses of 0.5–5 Gy, respectively, using a ^{60}Co γ-irradiator at a dose rate of 189.51 cGy/min. The aliquots without radiation were used as control group. After irradiation, the lymphocytes were separated from the irradiated whole blood in the same way (using the lymphocyte separation medium). Lymphocytes isolated before and after radiation were divided into two parts, one for CL detection, and the other for MTT assay.

CL analysis

After 30 min exposure to various doses of radiation (0.5–5 Gy), 1 mL of lymphocyte suspension (6×10^6 cells/mL) was added into the reaction cell. After 10 min

a CL probe, MCLA, was added into the cell suspensions. The concentration of MCLA was 5 or 8 μmol/L. Blood plasma irradiated with various doses was treated in a similar way. The effects of catalase, SOD, mannitol, D_2O and NaN_3 on MCLA-dependent CL of lymphocytes was observed by adding those reagents into parallel group irradiated with the same dosages.

MTT assay

Another sample of the lymphocyte suspension was cultured with PHA stimulation in 96-well microculture plates at 37°C, 5% CO_2, in RPMI 1640 medium supplemented with 15% bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin. After 3 days culture, the viability of lymphocytes was determined by the MTT method. The mortality of lymphocytes was calculated according to the inverse proportional relationship between cell mortality and cell viability.

RESULTS

The relationship between MCLA-dependent CL intensity of lymphocytes and radiation dose

CL detection results show no statistical difference between the lymphocytes isolated before and after radiation. The dose–response curve for MCLA-dependent CL intensity of lymphocytes (Fig. 3) indicates that with the increase of radiation dose, the MCLA-dependent CL increases gradually to a maximal intensity at a dose of 3 Gy, and then declines.

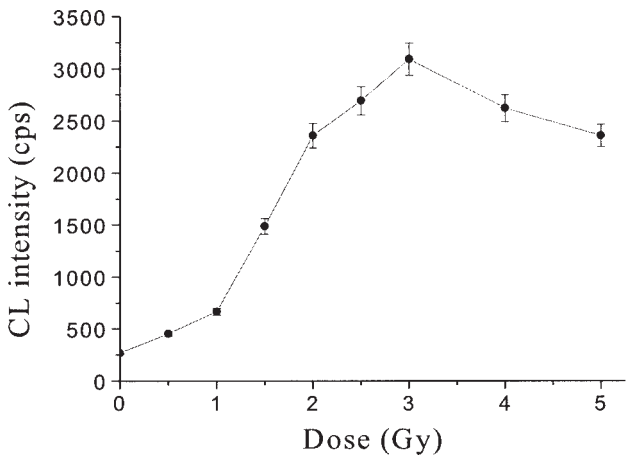

Figure 3. The dose–response curve for MCLA-dependent CL intensity of lymphocytes. The concentration of MCLA is 8 μmol/L. Dates are presented as mean ± SD of at least three separate experiments.

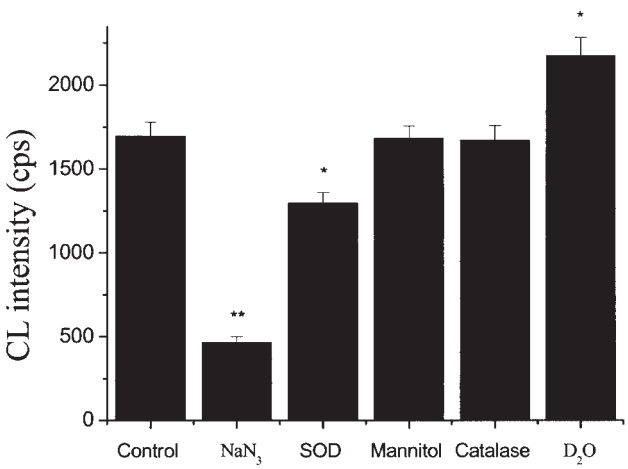

Figure 4. Effects of quenchers and D_2O on MCLA-dependent CL of lymphocytes. The irradiation dose is 3 Gy. The concentration of MCLA is 5 μmol/L. Dates are presented as mean ± SD of at least three separate experiments. $*p < 0.05$ in comparison with control; $**p < 0.01$ in comparison with control.

Effects of quenchers and D_2O on MCLA-dependent CL of lymphocytes

Figure 4 shows the effects of quenchers and D_2O on MCLA-dependent CL of lymphocytes. The irradiation dose was 3 Gy. We observed that the addition of the $^{\cdot}OH$ scavenger mannitol, and the H_2O_2 scavenger catalase, to the lymphocyte suspension did not cause the CL to decrease. However, CL intensity was markedly inhibited by the $O_2^{\cdot-}$ scavenger SOD (about 23.5%) and $^1O_2$ quencher NaN_3 (about 72.6%). The addition of D_2O, which can prolong the lifespan of $^1O_2$, caused an increase of CL (about 28%). Similar results were observed in lymphocytes irradiated at various other doses of radiation.

MCLA-dependent CL of plasma

Plasma exposed to various doses of radiation were tested by the CL method. Haemolysis occurred in the 5 Gy irradiation group, and so this data was excluded. MCLA-dependent CL intensities of all plasma samples were similar to that of the control group without irradiation.

Relationship between cell mortality of lymphocytes and radiation dose

The dose–response curve for cell mortality of lymphocytes indicated that the mortality of lymphocytes increased with an increase in the radiation dose (see Fig. 5).

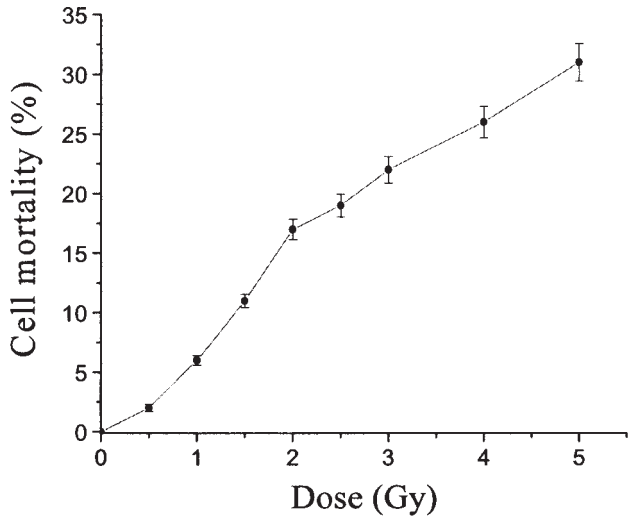

Figure 5. The dose–response curve for cell mortality of lymphocytes. Dates are presented as mean ± SD of at least three separate experiments.

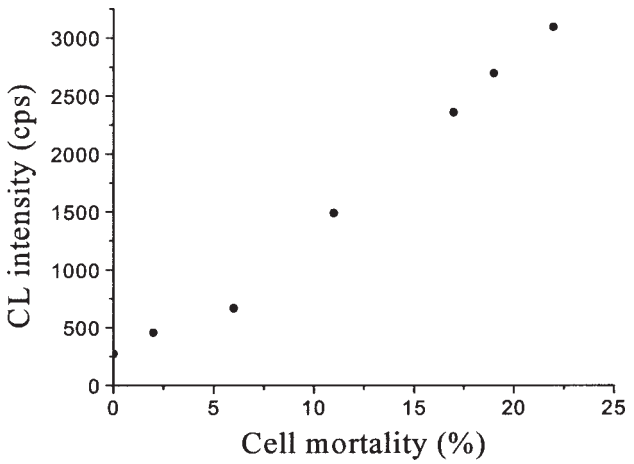

Figure 6. The relationship between cell mortality of lymphocytes and MCLA-dependent CL intensity.

Relationship between cell mortality of lymphocytes and MCLA-dependent CL intensity

Figure 6 indicates that the cell mortality of lymphocytes is positively related to MCLA-dependent CL intensity when the radiation dose is no more than 3 Gy, suggesting a positive relationship between the degree of damage of the lymphocytes and the amount of irradiation-induced ROS.

DISCUSSION

ROS are formed by incomplete reduction of molecular oxygen. They include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$) (16). The main site of ROS production in the cell is the organelle with highly oxidizing metabolic activities or with sustained electron flows, e.g. mitochondria (17). Low levels of ROS regulate cellular signalling and play an important role in normal cell proliferation (18, 19). In contrast to their role in promoting cell growth under non-stress conditions, ROS appear to activate and modulate apoptosis when cells are under stress. ROS levels are increased in cells exposed to various stress agents, including radiation, and they promote apoptosis by stimulating pro-apoptotic signalling molecules, such as ASK1, JNK and p38 (20–22). In addition, ROS can act directly on the apoptotic machinery, by accelerating mitochondrial depolarization and dysfunction during the effector phase of apoptosis (23, 24). The reactive nature of ROS makes them harmful to many cellular components, such as cell membranes, protein, DNA and phospholipid. Fortunately, organisms have the capacity to eliminate ROS by an efficient ROS-scavenging system. Under normal conditions, ROS are efficiently scavenged by the antioxidant defence system. However, during periods of some severe condition the scavenging system may become saturated by the increased rate of ROS production. Excessive levels of ROS result in damage to the mitochondria and cytomembranes, and ultimately lead to severe cellular injury, even death (17).

Radiation injury to living cells is, to large extent, due to oxidative stress (25, 26). ROS and free radicals induced by partial reduction of oxygen (O_2) react with cellular macromolecules (i.e. nucleic acids, lipids, proteins, and carbohydrates) (27) and damage them. The interaction of ionizing radiation with living cells induces a variety of reaction products and a complex chain reaction, in which many macromolecules and their degradation products participate (28). The amount of generated ROS caused by irradiation is related to the radiation dose (14). Ogawa proposed that the origin of hyper-radiosensitivity of lymphocytes seemed to be the high production of ROS in the mitochondrial DNA following irradiation (29).

According to the principle of cell stress response to ionizing radiation, we developed an effective MCLA-dependent CL method to analyse the degree of irradiation-induced lymphocyte damage by detecting the amount of ROS generated by lymphocytes. MCLA was used as a specific CL probe for O_2^- and $^1O_2$. The intensity of MCLA-dependent CL reflects the amount of ROS. The dose–response curve for MCLA-dependent CL intensity of lymphocytes (Fig. 3) indicated that the amount of the generated ROS was positively related to the radiation dose when the dosage was no more than 3 Gy. At higher doses of radiation, a small decrease was observed in CL intensity, suggesting that necrosis might become more predominant in lymphocytes after a certain dose.

The inhibition of CL by $^1O_2$ quencher NaN_3 and $O_2^{\cdot-}$ scavenger SOD confirmed the effect of $^1O_2$ and $O_2^{\cdot-}$

on MCLA-dependent CL (see Fig. 4). The solvent D_2O is used for confirming the effect of $^1O_2$ on MCLA-dependent CL, since the lifetime of $^1O_2$ is approximately 10 times longer in D_2O than in H_2O, thus allowing it more time to exert its effects on targets. In our experiments, the effect of generation of $^{\cdot}OH$ and H_2O_2 caused by irradiation cannot be assessed because of the selectivity of MCLA.

CONCLUSION

In this study, we demonstrate the first use of a MCLA-dependent CL method to detect γ-irradiation-induced ROS generated by lymphocytes. The results indicate that both CL intensity and cell mortality of lymphocytes increase with the increase of radiation dose when the dosage is no more than 3 Gy, suggesting a positive relationship between degree of cell damage of the lymphocytes and the amount of ROS generated. The CL method, thus, could potentially provide an easy way for evaluating lymphocytes damage caused by radiation.

Acknowledgements

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