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Low concentration of exogenous carbon monoxide protects mammalian cells against proliferation induced by radiation-induced bystander effect

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ABSTRACT

Radiation-induced bystander effect (RIBE) has been proposed to have tight relationship with the irradiation-caused secondary cancers beyond the irradiation-treated area after radiotherapy. Our previous studies demonstrated a protective effect of low concentration carbon monoxide (CO) on the genotoxicity of RIBE after α-particle irradiation. In the present work, a significant inhibitory effect of low-dose exogenous CO, generated by tricarbonyldichlororuthenium (II) dimer [CO-releasing molecule (CORM-2)], on both RIBE-induced proliferation and chromosome aberration was observed. Further studies on the mechanism revealed that the transforming growth factor β1/nitric oxide (NO) signaling pathway, which mediated RIBE signaling transduction, could be modulated by CO involved in the protective effects. Considering the potential of exogenous CO in clinical applications and its protective effect on RIBE, the present work aims to provide a foundation for potential application of CO in radiotherapy.

1. Introduction

Radiation-induced bystander effects (RIBE), a phenomenon in which the irradiated cells release some signaling molecule(s) which are transferred *via* the medium or cellular gap-junction to attack neighboring non-irradiated cells, thereby leading to cytotoxicity or genotoxicity similar to those found in irradiated cells. RIBE was extensively investigated in the past decade [1]. *In vitro* and *in vivo* research revealed that a significant increase of gene mutations [2,3], DNA damage [4,5], cell proliferation [6,7], chromosomal damage [8,9], neoplastic transformation [10] and even tumor formation [11] could be induced by RIBE. The occurrence of RIBE has potential hazard to normal tissues surrounding the targeted region in radiotherapy, and RIBE was considered to have tight relationship with the radiation-induced secondary cancers beyond the irradiated area after radiotherapy [1,12].

The sustained proliferation, gene mutations and chromosomal instability were considered risk factors for tumorgenesis [13,14]. RIBE-induced biological effects, especially proliferation and chromosomal abnormality in non-irradiated cells or tissues, increase the possibility of neoplastic transformation of cells after radiotherapy. In previous researches, chemicals including Vitamins C and E [15,16] *etc.* were used to protect cells from RIBE.

Carbon monoxide (CO) at low concentration could modulate many cell signaling pathways, and exogenous CO had potential uses in clinical therapy of diseases such as inflammatory diseases of the lung in humans [17]. Low-concentration CO has been shown to exert biological functions as diverse as protection against cell death, anti-inflammatory effects, protection against oxidative injury, inhibition of cell proliferation, neurotransmission and tolerance of organ transplantation [17]. The protective effect of exogenous CO against the genotoxicity of RIBE was proved in our previous studies [18,19]. Exogenous CO at relative low concentration (14 μM) attenuated RIBE-induced DNA double-strand breaks (DSBs) and chromosome breaks (micronucleus, MN) *via* decreasing the excessive $O_2^{\bullet -}$ in non-irradiated bystander cells, and no significant changes were observed in the irradiated cells [18,19].

In the present study, we investigated the effect of low concentrations CO on a RIBE system, *i.e.*, an increase of chromosome aberration in proliferating bystander Chinese hamster ovary (CHO)

Abbreviations: AG, aminoguanidine hemisulfate salt; CBMN, cytokinesis-B blocked micronucleus; CHO, Chinese hamster ovary; CO, carbon monoxide; CORM, CO releasing molecule; COX-2, cyclooxygenase-2; DAF-FM, 4-amini-5-methylamino-2′,7′-difluorofluorescein diacetate; DCF, 2′,7′-difluorofluorescein diacetate; DMSO, dimethyl sulfoxide; DSBs, DNA double-strand breaks; HO, heme oxygenase; MN, micronucleus; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; NPB, nucleoplasmic bridges; RCG, relative cell growth; RIBE, radiation induced bystander effect; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α.

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cells, and the possible mechanisms were also further studied. The present results could provide some hints for potential applications of CO in radiotherapy.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells were cultured in a mixed medium Dulbecco's Modified Eagle Medium: F-12 = 1:1 (Invitrogen, Grand Island, NY) supplied with 10% fetal calf serum (Thermo scientific Hyclone, Logan, UT) and maintained at 37 °C under 95% air and 5% CO_2. The medium was replaced every 2 days. Cells under 70%-80% confluence were trypsinized and 3.0×10^5 cells were seeded into each specially designed stainless iron ring (inner diameter = 32 mm) holding a 3.5 μm Mylar film bottom. The cells were then incubated for 36 h for irradiation, the time at which the cells were under full confluence condition.

As for bystander cells, 2.0×10^5 CHO cells were seeded in each transwell insert (Millipore, Billerica, MA) with a 4.5 cm^2 growth area and pores (diameter: 1 μm) for 12 h before co-culture with the irradiated cells.

2.2. Irradiation and co-culture system

The average energy of α particles derived from an ^{241}Am irradiation source was 3.5 MeV at the cell layer, and the particles were delivered at a dose rate of 1.04 cGy/s. Before irradiation, the medium in each stainless iron ring and transwell insert dish was replaced with 2 ml and 1 ml fresh complete medium, respectively. Sham-irradiated samples were used as controls.

Immediately after irradiation, the transwell inserts were put into iron rings, and then the irradiated and non-irradiated cells were co-cultured for a 24 h for the further experiments.

2.3. CO treatment

CO was generated by the CO releasing molecule, $[Ru(CO)_3Cl_2]_2$ (CORM-2, Sigma–Aldrich, St. Louis, MO), which released CO when dissolved in the medium. For each mole of CORM-2, 0.7 mole of CO was liberated [20]. The stock solution (50 mM) was freshly prepared by dissolving CORM-2 in dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO). The cell populations (both irradiated and non-irradiated bystander populations) were treated with or without CORM-2 for 1 h before irradiation and the chemical would be present in the culture system until the end of co-culture. Control experiments were performed by using $RuCl_3$ instead of CORM-2 dissolved in DMSO.

2.4. Drugs treatment

In some of the present experiments, the cells were treated with a specific inducible NOS (iNOS) inhibitor, aminoguanidine hemisulfate salt (AG, Sigma–Aldrich, St. Louis, MO), at final concentration of 1 mM or monoclonal anti-human TGF-β1 (Santa Cruz, Dallas, Texas) at final concentration of 10 μg/ml during and after irradiation to investigate the role of NO and TGF-β1 in RIBE.

Sper/NO (Sigma–Aldrich, St. Louis, MO) and human recombinant TGF-β1 (rTGF-β1, Prospec, Rehovot, Israel) were employed to clarify the possible mechanism of CO on RIBE.

The sper and rTGF-β1 were added to the medium at finial concentration of 20 μM and 5 ng/ml respectively.

2.5. Cell proliferation assay

After 24 h co-culture, the irradiated and non-irradiated bystander cells were harvested and 10^5 cells were seeded in 60 mm Petri dishes, respectively. The cell number was then counted with hemocytometer at an indicated time point after cell seeding.

2.6. MN and nucleoplasmic bridges (NPB) test

MN and NPB were scored with the cytokinesis-block technique [21]. The cells were trypsinized after co-culture, and then sub-cultured in 35 mm Petri dishes. The medium was replaced with fresh medium containing 1.5 μg/ml cytochalasin-B (Sigma–Aldrich, St. Louis, MO) at 6 h after cell seeding, and the cells were cultured for further 24 h. The cells were then fixed with 2% paraformaldehyde (Sinopharm Chmemical Reagent, Shanghai, China), stained with 0.01% acridine orange (Sigma–Aldrich, St. Louis, MO), and images were captured using a florescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan). The MN and NPB only in bi-nucleated (BN) cells were morphologically identified and more than 1000 BN cells were scored for each sample. The frequency of MN or NPB (r^0) was calculated as: $r^0 = a/b$, where a is the total number of MN or NPB cells scored, and b is the total of binucleated cells examined.

2.7. Measurement of TGF-β1 release

The relative content of TGF-β1, generated from irradiated CHO cells, in the co-culture medium was measured with human TGF-β1 ELISA kit (Senxiong, Shanghai, China) according to the protocol provided by manufacturer.

2.8. NO measurement in bystander cells

The intracellular NO level was measured with its specific fluorescence probe 4-amini-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM, Invitrogen, Eugene, OR). Briefly, cells growing in transwell inserts were harvested and treated with 20 μM DAF-FM diacetate for 30 min at 37 °C. The cells were then washed twice with cold Hanks' solution containing 1% fetal calf serum. To determine the DCF content in the cells, 0.02% digitonin (pH 7.2) was added immediately and the cells were incubated for 20 min at 37 °C. Subsequently, the medium was decanted and centrifuged for 5 min at $700 \times g$ and the samples were put on ice prior to measuring the DCF content with a microplate fluorescent reader (excitation/emission: 495/515 nm, Thermo, Vantaa, Finland) at 2 °C. Statistical analysis was performed on the means of the data pooled from at least three independent experiments.

2.9. Western blotting

SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described in Ref. [22]. Samples (40 μg of protein) were subjected to 12% SDS-PAGE, transferred onto PVDF membranes (Millipore, Billerica, MA) and assayed for CDC2 ($p34^{cdc2}$; 1:200; Santa Cruz Biotechnology) and β-tublin (Cell Signaling, Boston, MA) protein expression using chemiluminescence detection (Super ECL Plus Detection Reagent) according to the manufacturer's instructions. The relative levels of CDC2 protein were measured by densitometry and analyzed with software Image J.

2.10. Statistical analysis

Statistical analysis was performed on data obtained from at least three independent experiments. All results are presented as means ± SD. Significance level is assessed using Student's t-test and