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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

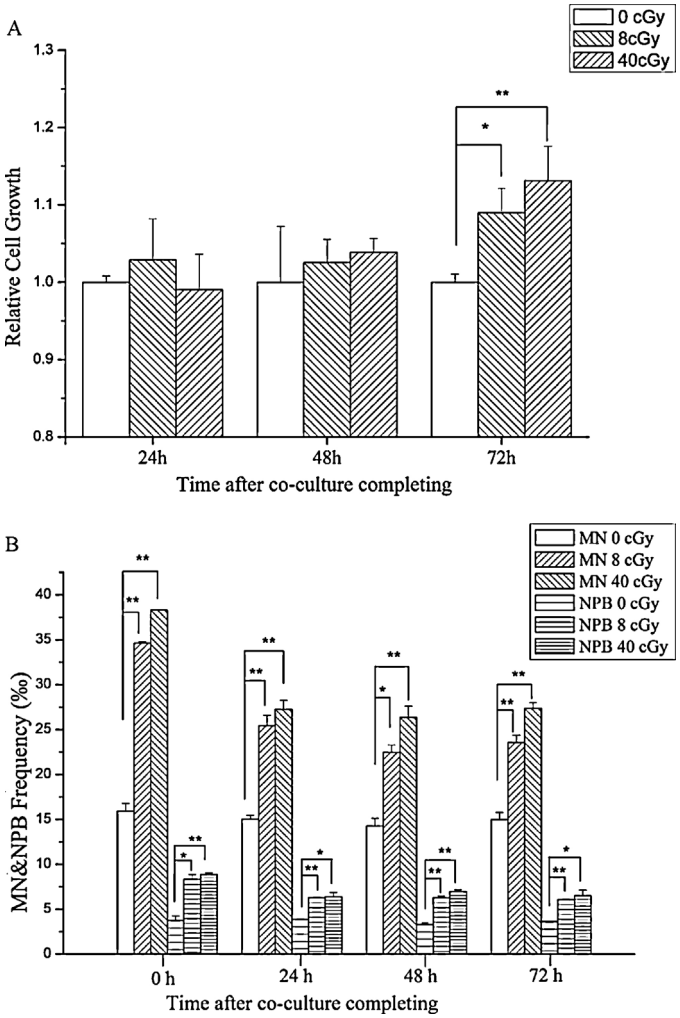

Fig. 1. (A) Relative cell growth (RCG) and (B) MN/NPB formation in bystander cells after co-cultured with cells irradiated at 0, 8 or 40 cGy. Data are pooled from at least 3 independent repeats and the results are presented as mean ± SD. Significances in the differences between the samples are determined and differences with $p < 0.05$ are considered statistically significant ($^{*}p < 0.05$; $^{**}p < 0.01$).

differences corresponding to $p < 0.05$ are considered statistically significant.

3. Results

3.1. RIBE induces chromosome aberration and proliferation simultaneously in bystander cells.

Relative cell growth (RCG), defined as the ratio between the numbers of treated and control cells, was used to quantify the proliferation of bystander cells at the specified time point after completing co-culture with irradiated cells. The results in Fig. 1A showed that RCG of bystander cells had no significant increase at 24 h and 48 h compared with sham-irradiated control. However, at 72 h, RCG value significantly increased to ~1.09 and ~1.13-fold of the control after 8 and 40 cGy irradiation, respectively.

The chromosome aberration (MN and NPB) was also assessed in the proliferated bystander cells (Fig. 1B). Although the results indicated time dependence of the repair of chromosome damages, significantly increased frequencies of MN and NPB were detected at 24, 48 and 72 h after the co-culturing of cells. It was noticed that significant MN and NPB yields still existed at 72 h, with increases of 57.2% and 68.5%, respectively, in the 8 cGy irradiated group, when the bystander cells were proliferating considerably. In subsequent experiments, the time point of 72 h after cell co-culture was chosen to study the effect of CO treatment.

3.2. CO (CORM-2) treatment decreases proliferation and chromosome aberration in bystander cells

The inhibiting effect of CO on proliferation of bystander cells showed a concentration-dependent manner. The RCG level was decreased to the background level after treatment with 20 μM CORM-2 (Fig. 2A).On the other hand, the protein CDC2, a catalytic subunit of a protein kinase complex called the M-phase promoting factor, was found to be highly expressed in proliferating bystander cells treated with the conditioned medium harvested from low-dose α-particle irradiated cells [38]. The level of COD2 protein was also found to have reduced from 1.62-fold to 0.82-fold of the control after treatment with 20 μM CORM-2 (Fig. 2B). Nevertheless, no significant effect of CO was observed in irradiated cells (data not shown).

As regards RIBE-induced chromosome aberration (MN and NPB), CO treatment also showed a distinct attenuating effect and even 10 μM of CORM-2 effectively reduced the yields of MN and NPB to the background level (Fig. 2C). Similarly, no distinct effect of CO treatment on chromosome aberration (MN and NPB) was observed in irradiated cells (data not shown).

3.3. TGF-β1/NO signaling pathway mediates RIBE transduction

To investigate the possible signaling pathway involved in RIBE-induced proliferation and chromosome aberration, TGF-β1 antibody and AG, a specific inhibitor of iNOS, were employed. As shown in Fig. 3A and B, both proliferation and MN formation of bystander cells decreased distinctly with TGF-β1 antibody or AG treatment. This result indicated that TGF-β1 and NO played important roles in inducing these effects.

Notably, the RCG of cells in both irradiated and non-irradiated groups were decreased after treated with TGF-β1 antibody. This was expected since TGF-β1 was a multifunctional peptide which controlled proliferation. Application of the TGF-β1 antibody reduced the combination of TGF-β1 receptor with TGF-β1 and therefore decreased the proliferation rate of cells.

Significant release of TGF-β1 in the medium and distinctly elevated NO content in bystander population were also detected after 8 cGy irradiation (Fig. 4A and B). The TGF-β1 and NO level were increased to about 1.24 and 2.15 folds the control level after 24 h co-culture, but this level decreased to the background level with the treatment of CO.

To mimic the TGF-β1/NO mediated RIBE transduction, rTGF-β1 or sper, a generator of NO, were adopted. The results, consistent with previous study [7,23], showed that increased RCG and MN formation (Fig. 5A and B) were observed.

3.4. CO modulates TGF-β1/NO signaling pathway

Fig. 4A illustrated that the elevated release level of TGF-β1 in bystander cells was markedly reduced to the background level after treatment with 20 μM CORM-2. Similarly, NO formation in bystander cells was also significantly decreased with the same treatment (Fig. 4B).

In the mimic experiment, CO treatment also effectively attenuated RCG and MN yields induced by rTGF-β1 and NO (sper), which were used to simulate TGF-β1/NO- mediated RIBE transduction (Fig. 5A and B). Combined these results together, CO might protect the bystander cells *via* modulating the TGF-β1/NO signaling pathway.

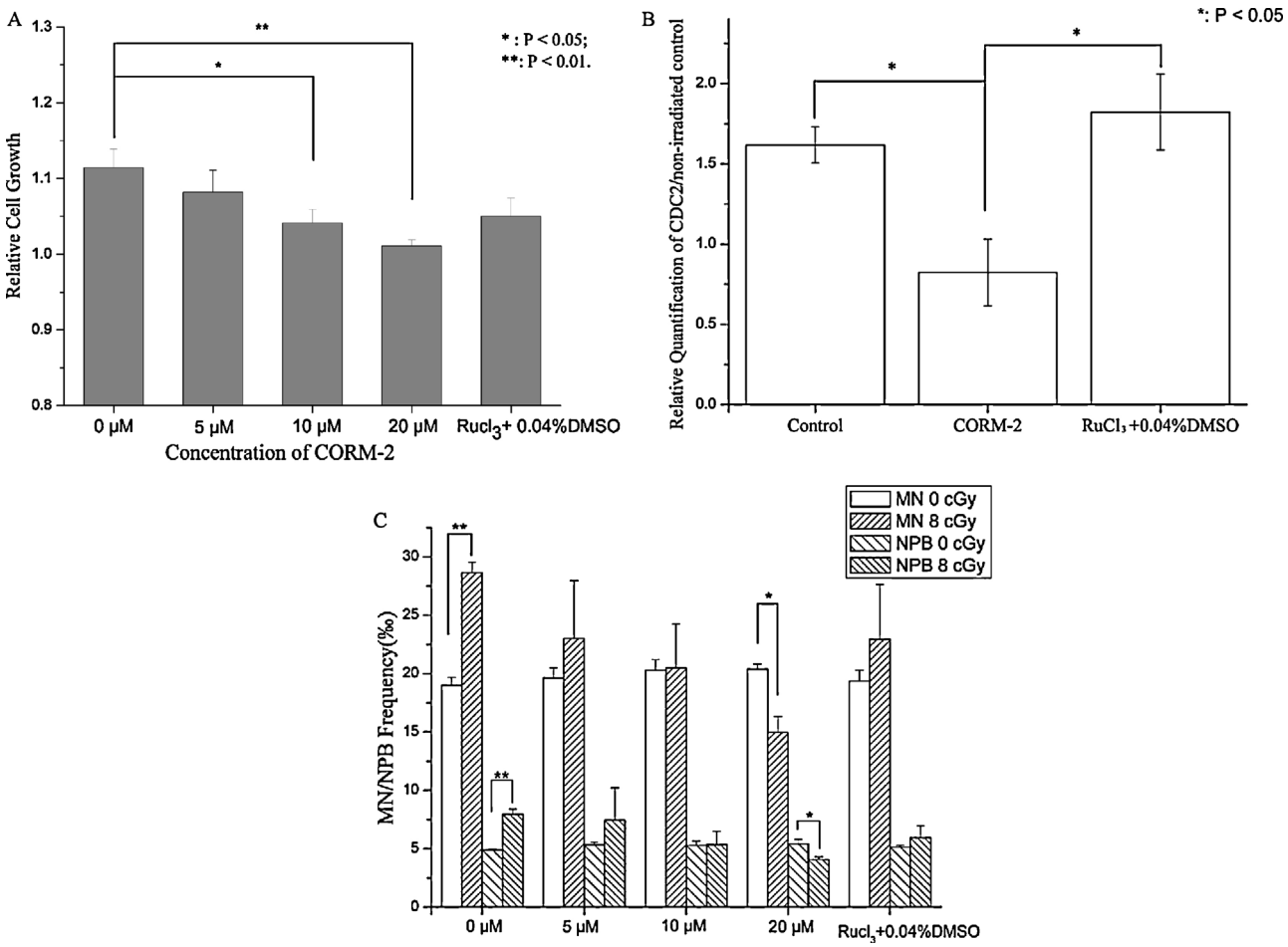

Fig. 2. CO decreases cell proliferation and chromosome aberration (MN and NPB) in bystander cells. (A) RCG of bystander cells with treatment of CORM-2 after irradiation. (B) Levels of CDC2 expression in bystander cells analyzed by Western blotting and the relative quantification obtained using Image J. (C) MN and NPB yields in bystander cells with treatment of CORM-2. Data are pooled from at least 3 independent repeats and the results are presented as mean ± SD. Significances in the differences between the samples are determined and differences with $p < 0.05$ are considered statistically significant ($^{*}p < 0.05$; $^{**}p < 0.01$).

4. Discussion

The present results indicated that a low concentration (14 μM) of exogenous CO could effectively inhibit the RIBE-induced cell proliferation and chromosomal abnormality (MN and NPB). Exogenous CO also protected cells against the toxicity of sper (the NO generator) and TGF-β1 which were used to mimic the situation in bystander cells. The study on mechanisms indicated that CO could reduce the TGF-β1 released by irradiated cells and further reduce the NO production in bystander cells.

RIBE has been deemed to have close relationship with radiation-induced secondary cancer beyond the irradiated area after radiotherapy, and RIBE was not dependent on the type of irradiated and bystander cells. RIBE signaling could be transmitted from an irradiated tumor cell population to a normal or tumor bystander cell population [1]. Shao et al. irradiated the human glioblastoma T98G cells and detected increased MN formation in bystander normal human primary fibroblast AG 1522 cells or T98G cells [9]. In the present work, CHO cells were employed the RIBE study [1,24].

In corroboration with previous studies [6,7,23] our results showed that increased cell proliferation and chromosome abnormality (MN and NPB) were simultaneously induced by RIBE in a co-culture system. MN represented the unrepaired DSBs in DNA and included chromosomes without centromeres (acentric fragments) and whole chromosomes, which could be measured in the cytokinesis-B blocked micronucleus (CBMN) assay. NPB was an important biomarker of chromosome abnormality, and originated from dicentric chromosomes which were pulled to opposite poles of the cell at the anaphase [25,26]. Notably, chromosomal abnormality existed in sustained proliferating bystander cells. These results indicated that the probability of neoplastic transformation of the normal tissues surrounding the irradiated area, where cell proliferation and chromosome abnormality were induced by RIBE in radiotherapy, might increase after radiotherapy [11,13,14].

Previous studies on the mechanisms of RIBE revealed that soluble factors and oxidative stress (ROS/NO production) played crucial roles in mediating RIBE signaling transduction [2,7,9]. There were extensive evidence suggesting the important role of TGF-β1 and NO in RIBE-induced proliferation and genotoxity [23,27]. Iyer and Lehnert detected an elevated level of TGF-β1 in supernatants harvested from irradiated normal human lung fibroblasts, and this was directly related to the proliferation of non-irradiated bystander cells [6]. Our previous study also indicated that treatment with TGF-β1 antibody could inhibit the proliferation in bystander cells mix co-cultured with irradiated cells [28]. Shao et al. studied the function of NO and TGF-β1 in RIBE transduction, and the "NO-TGF-β1-NO" pathway was proposed [7,9,23]: NO was generated through activation of iNOS in the irradiated cells, possibly mediated by the sphingomyelin signal-transduction pathway in the membrane, and the irradiated cells released TGF-β1 as a result of the elevated level of NO; the released TGF-β1 diffused through the medium and acted as an extracellular signaling factor to "attack" the bystander cells;

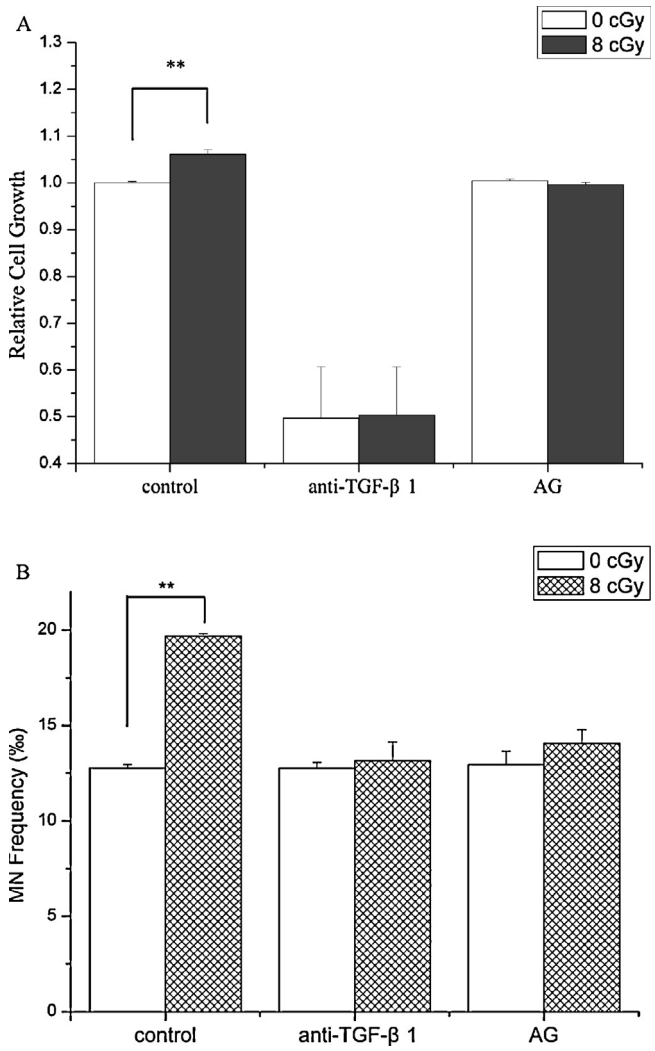

Fig. 3. (A) RCG and (B) MN yields of bystander cells with treatment of TGF-β1 antibody and iNOS inhibitor (AG). Data are pooled from at least 3 independent repeats and the results are presented as mean ± SD. Significances in the differences between the samples are determined and differences with $p < 0.05$ are considered statistically significant ($^{*}p < 0.05$; $^{**}p < 0.01$).

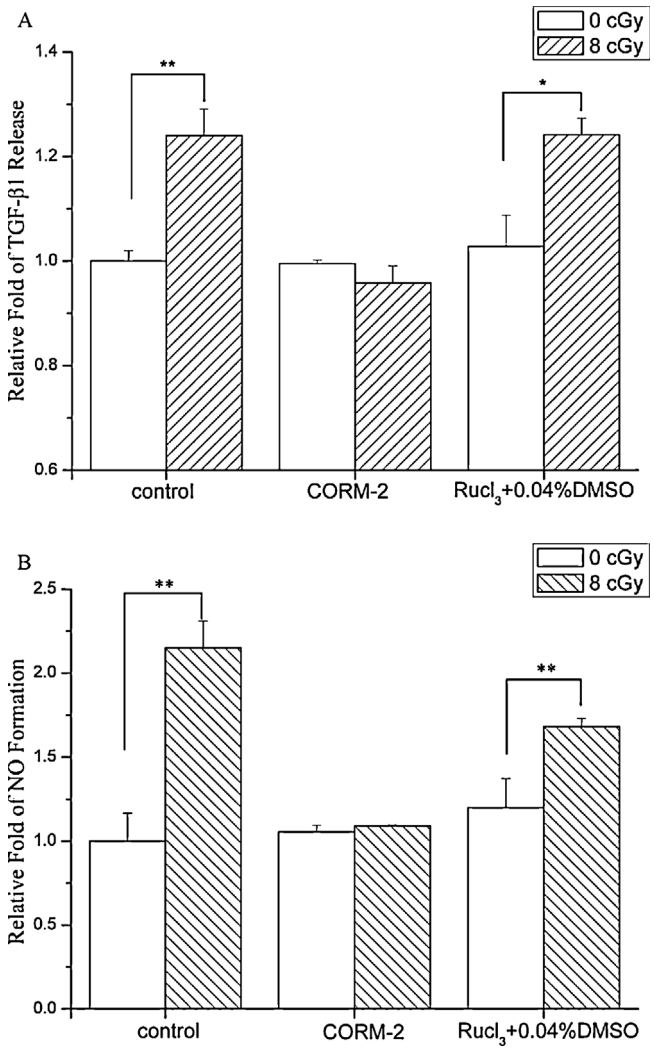

Fig. 4. (A) Relative release of TGF-β1 in the co-culture medium and (B) relative NO production in the bystander cells after irradiation and CORM-2 (20 μM) treatment. Data are pooled from at least 3 independent repeats and the results are presented as mean ± SD. Significances in the differences between the samples are determined and differences with $p < 0.05$ are considered statistically significant. ($^{*}p < 0.05$; $^{**}p < 0.01$).

the released TGF-β1 led to generating NO in the bystander cells, probably *via* a calcium-dependent pathway, and further caused chromosome breaks (MN formation) in the bystander cells [7,9,23]. The results in the present study also indicated an elevated release of TGF-β1 by the irradiated CHO cells and significant NO production in the bystander cells. By treatment with TGF-β1 antibody or NOS inhibitor, the RIBE-induced proliferation and MN formation were inhibited. These indicated that the TGF-β1/NO pathway also mediated RIBE transduction in the co-culture system adopted in the present study.

CO, a product of heme catabolized by heme oxygenase (HO), could protect cells or tissues against ROS/NO stress and inflammatory injury [17]. Li et al. reported the protection of CO induced by HO-1–PC 12 cells from nitrosative stress through the increased glutamate-cysteine ligase induced by CO [29], with the understanding that glutamate-cysteine ligase was important in glutathione biosynthesis and glutathione was an important antioxidant for the maintenance of intracellular redox balance [29]. Other results revealed that HO-1 and CO might modulate NO signaling to defend against the action of NO through reducing the expression of NO synthase or limiting the function of NO synthase [30]. In addition, recent research demonstrated that low concentration of CO could attenuate the increased level of superoxide anion in bystander cells [18] possibly through modulation of NADPH oxidase [31] or upregulation of glutamate-cysteine ligase expression [29]. Abdel-Aziz et al. reported that overexpression of HO-1 attenuated TGF-β production in rat lung microvessel endothelial cell line in the presence of HO-1 inducers [32].

The potential of clinical application of CO at low concentrations was previously demonstrated by treating inflammatory diseases, lung injuries, cardiovascular injuries *etc.* with administering CO for inhalation or using CORMs [17]. The present study aimed to provide a foundation for future employment of low-concentration CO to protect the normal tissues beyond the irradiated area against the risk of secondary cancer caused by RIBE in radiotherapy.

CO might also affect the other reported RIBE signaling pathways such as the cyclooxygenase-2 (COX-2) signaling pathway [33,34]. Activation of NF-κB, a very important transcription factor in COX-2 signaling pathway, could be attenuated by treatment with CO in the inflammatory tissue [35]. Our previous study also revealed that CO could prevent the production of superoxide anion *via* inhibiting the activation of NADPH oxidase in bystander cells [18]. CO could also inhibit the production of pro-inflammatory cytokines,

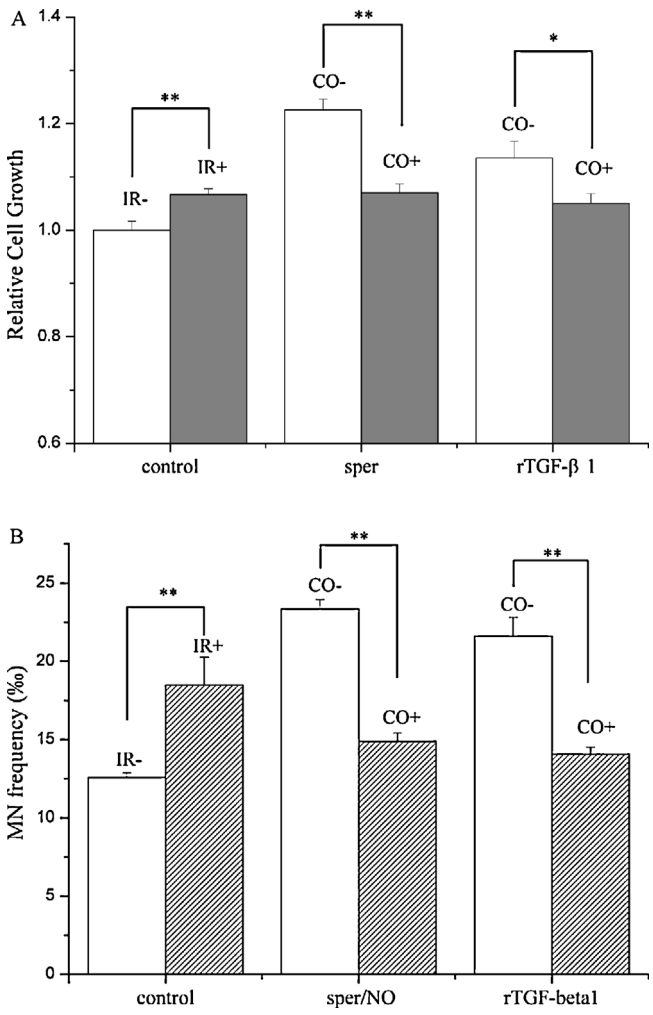

Fig. 5. (A) Inhibitory effect of CORM-2 (20 μM) on TGF-β1 and NO-induced cell proliferation, and (B) MN formation. Data are pooled from at least 3 independent repeats and the results are presented as mean ± SD. Significance in the differences between the samples is determined and differences with $p < 0.05$ are considered statistically significant. ($^{*}p < 0.05$; $^{**}p < 0.01$).

such as tumor necrosis factor-α, interleukin-1 and could increase production of anti-inflammatory cytokine interleukin-10 through the p38 mitogen-activated protein kinase-dependent pathway, and could thus exhibit important cytoprotective function [36,37]. These cytokines and the associated pathways had been suggested to play very important roles in RIBE transduction [1].

Conflict of interest statement

The authors declare that they have no competing interests.

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