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Exogenous carbon monoxide protects the bystander Chinese hamster ovary cells in mixed coculture system after alpha-particle irradiation

Wei Han, Lijun Wu[1], Shaopeng Chen[1] and K.N.Yu*

Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong and [1]Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, Hefei 230031, People's Republic of China

*To whom correspondence should be addressed. Tel: +852 27887812; Fax: +852 27887830;
Email: peter.yu@cityu.edu.hk

In the present work, the inhibitory effect of carbon monoxide (CO), generated by tricarbonyldichlororuthenium (II) dimer [CO-releasing molecule (CORM-2)], on the toxicity of radiation-induced bystander effect (RIBE) after α-particle irradiation was studied in a mixed coculture system. CO (CORM-2) treatment showed a significant inhibitory effect to the formation of p53 binding protein 1 (BP1) and micronuclei (MN) induced by RIBE in a concentration-dependent manner, but in the directly irradiated cell population no distinct decreases of BP1 and MN formation were observed. In this mixed coculture system, nitric oxide (NO) or superoxide anion ($O_2^{\cdot-}$) was also proved to mediate the transduction of RIBE by using a NO synthase inhibitor or NADPH-oxidase-specific inhibitor treatment. The elevated $O_2^{\cdot-}$ was attenuated by CO (CORM-2) treatment in the bystander cells as measured by hydroethidine staining and fluorescence assessment. The exogenous NO (sper) or $O_2^{\cdot-}$ (H_2O_2) was used to mimic NO/O_2-mediated RIBE, and CO (CORM-2) treatment also showed a protective effect to cells against the toxicity of these exogenous factors. Considering the inhibitory effect of CO on RIBE and the wide use of CO in therapy of diseases, it is hoped that a low concentration of CO can protect normal tissues against RIBE during radiotherapy.

Introduction

Radiation-induced bystander effect (RIBE) has been studied widely since its revelation by Nagasawa *et al.* (1) in 1992. Accumulating evidences have indicated that the irradiated cells might release some stress signal factor(s) to affect the cells nearby or to affect the cells that have received the medium conditioned by the irradiated cells. The signaling factors cause excessive DNA damages, expression of DNA damage-related proteins, chromosome aberration, mutation, decreased cell viability, malignant transformation, etc. The observations of RIBE have challenged the conventional dogma of radiation protection, and it increases the cancer risk of environmental low-dose irradiation to human beings (reviewed in refs 2,3). This phenomenon was not only observed in various types of cultured cells but also in a three-dimensional tissue (4,5). Studies in mouse models showed RIBE-induced DNA damages and methylation in cutaneous tissue (6) and even tumor induction in cerebellum (7) beyond the local area exposed to irradiation. Shao *et al.* (8) reported that irradiation of human glioblastoma T98G cells significantly induced excessive production of micronuclei (MN) and nitric oxide (NO) in the cocultured human primary skin fibroblast AG 1522. All these studies indicated that in tumor radiotherapy the surrounding normal tissues might be 'attacked' by the bystander effect signal(s) released by the irradiated tumor tissues, and RIBE enhanced the cancer risk of normal tissue after the radiotherapy. Therefore, it is necessary to develop strategies to minimize the effect of RIBE in normal tissues in radiotherapy. Antioxidants such as Vitamins C or E were firstly studied to protect cells from RIBE (9).

Carbon monoxide (CO) at low concentrations 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 while being toxic and lethal to living organisms at high concentrations (reviewed in ref. 10). Endogenous CO can be produced mainly by heme oxygenase (HO), an enzyme that catalyzes the oxidization of heme, in the body, and CO performs various physiological functions in the circulatory, nervous, respiratory, reproductive, gastrointestinal systems and various organs (reviewed in ref. 11). The exogenous application of CO produces similar effects as HO in many models. In the *HO-1*-deficient cells, exogenous CO is able to suppress the excessive generation of reactive oxygen species (ROS) and expression of plasminogen activator inhibitors, leading to preservation of viability (12). Low concentration of CO protected endothelial cells against hyperoxia-induced apoptosis by inhibiting NADPH-oxidase-dependent ROS formation (13). Exogenous CO could attenuate the production of ROS/NO and cytokines, as well as activation of nuclear factor-kappaB (NF-κB) in lipopolysaccharide-induced inflammation to protect the lung and the liver (14). Further studies showed that CO differentially regulated the expression of inducible NO synthase/NO in different tissues to act as a component of the protective system in those organs (15). Inhaling CO or employing CO-releasing molecule (CORM) have been demonstrated to be effective in animal models against inflammation, hypertension, rejection of organ transplantation, vascular injury and ventilator-induced lung injury, and gas inhalation strategies can be used for therapies for inflammatory diseases of the lung in humans (reviewed in ref. 10).

In the present study, we demonstrated that pretreatment of bystander Chinese hamster ovary (CHO) cells with CO released by CORM-2, ticarbonyldichlororuthenium (II) dimer [$(Ru(CO)_3Cl_2)_2$], would lead to a reduction in the formation of p53 binding protein 1 (53BP1) foci and MN, which were induced by the bystander effect after 10 cGy α-particle irradiation, in a concentration-dependent manner in a mixed coculture system. No significant influence of CO (CORM-2) was detected in the irradiated population. Further studies showed that RIBE increased the level of superoxide anion ($O_2^{\cdot-}$) in bystander cells and that CO (CORM-2) treatment significantly attenuated the increased level of $O_2^{\cdot-}$. H_2O_2 or NO generator sper were used to treat cells to mimic the ROS- or NO-mediated RIBE, and pretreatment with CO (CORM-2) was found to be able to effectively protect the CHO cells against DNA or chromosomal damages [viz, formation of binding protein 1 (BP1) foci or MN] caused by chemicals treatment. Through the inhibitory effect of CO (CORM-2) on the bystander effect, a low concentration of CO (CORM-2) has a potential use in radiotherapy to protect normal tissues against the bystander effect signal(s) released by the irradiated tumor tissues.

Abbreviations: 53BP1, p53 binding protein 1; BP1, binding protein 1; CHO, Chinese hamster ovary; CO, carbon monoxide; CORM, CO-releasing molecule; COX-2, cyclooxygenase-2; DSB, double-strand break; HE, hydroethidine; HO, heme oxygenase; MN, micronuclei; NF-κB, nuclear factor-kappaB; NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; PBS, phosphate-buffered saline; RIBE, radiation-induced bystander effect; ROS, reactive oxygen species.

Materials and methods

Cell culture

CHO-K1 cells were cultured in modified eagle medium (Gibco, Grand Island, NY): F-12 (Gibco) (1:1) mixed medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 2 days. Cells under 70–80% confluence were trypsinized and 5×10^4 cells were seeded into each specially designed stainless iron ring (inner diameter = 9.0 mm) holding a 3.5 μm mylar film bottom. The cells were then incubated for 24 h for irradiation, the time at which

the cells were under full confluence condition. In addition, 6×10^4 cells were seeded into $D = 35$ mm Petri dishes at the same time. The cells were then incubated for 24 h, at which time the number of cells was 2×10^5, to act as bystander cells.

Alpha-particle irradiation and mixed coculture system

The average energy of α-particles derived from an ^{241}Am irradiation source with an activity of 1.86×10^5 Bq was 5.16 MeV at the cell layer, and the α-particles were delivered at a dose rate of 0.477 cGy/s.

A mixed coculture system was employed to study the bystander effect induced by α-particle irradiation. Gerashchenko *et al.* (16–19) used this method to assess the proliferative effect of RIBE. Briefly, before irradiation, the irradiated cells were stained with 5 μM CellTracker Orange CMRA (Invitrogen, Eugene, OR) in the culture medium for 30–45 min and then washed with phosphate-buffered saline (PBS) twice to remove the excessive dye. After irradiation, the irradiated population was trypsinized and 5×10^4 irradiated cells were transferred into the dishes in which the bystander cells were seeded beforehand and cocultured with the bystander cells in a mixed manner for a chosen time period. In this mixed coculture system, the ratio of irradiated and bystander cells were 1:4 and the fluorescence of CellTracker Orange CMRA could distinguish the irradiated (with fluorescence) from the bystander cells (with no fluorescence). CellTracker Orange CMRA passes freely through cell membranes but is transformed into cell-impermeable fluorescent reaction products once inside the cell. The fluorescent tracer can be inherited by daughter cells after cell divisions and will not be transferred to adjacent cells in a population (20). This kind of staining dyes has been widely used to distinguish between the irradiated or non-irradiated cells in microbeam irradiation experiments (21,22). It has been demonstrated that this fluorescent tracer has no toxicity to cells and does not affect DNA damage repair and cell proliferation (20,21).

CO (CORM-2) treatment

In the present experiments, CO was generated by $(Ru(CO)_3Cl_2)_2$ (Sigma, St Louis, MO), which is a CORM-2. This chemical can release CO when dissolved in the medium and for each mole of CORM-2, 0.7 mole of CO will be liberated (23). The stock solution (50 mM) was freshly prepared by dissolving CORM-2 in dimethyl sulfoxide (Sigma). The cell populations were then treated with CORM-2 1 h before irradiation or other drug treatment, and the chemical would be present in the culture until cell fixing (14). Control experiments were performed by using $RuCl_3$ instead of CORM-2 dissolved in dimethyl sulfoxide. To confirm the protective effect of CO released by CORM-2, hemoglobin was used to treat the culture. Hemoglobin (Sigma) is a scavenger of CO (23,24). Hemoglobin stock solution was mixed with a CORM-2 solution or a CORM-2 absent solution, which were then added into the culture medium to treat the cells. The final concentration of hemoglobin was 20 μM. Hemoglobin was present in the medium until the cells were fixed for further experiments.

Immunofluorescence of 53BP1 and DNA double-strand break measurement

53*BP1* is a member of the BRCT (BRCAI C-terminal) repeat family, which consists of many members, including the DNA damage response proteins NBS1 and BRCA1. 53BP1 is required for the phosphorylation of numerous ataxia-telangiectasia-mutated substrates during the double-strand break (DSB) response (25,26). 53BP1 has been shown to relocalize into foci shortly after irradiation, with the number of foci closely paralleling the number of DNA DSBs (27).

In this work, we employed fluorescent detection of foci formation using 53BP1 as a marker of DNA damages (28). Immunochemical staining of cells was performed as described (29). Briefly, the cells were fixed in 2% buffered paraformaldehyde (Sigma) at chosen time points after cell mixture for 15 min at room temperature and were then rinsed three times with PBS again. Prior to immunochemical staining, the cells were incubated for 30 min in TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% fetal bovine serum) to improve their permeability. The cells were then incubated with rabbit anti-53BP1 antibody (Abcam, Cambridge, MA) at 1:200 in PBS^+ (PBS supplemented with 1% fetal bovine serum) for 90 min, washed in TNBS for 3×5 min and incubated in PBS^+ containing secondary anti-rabbit Alex fluor-488 (Invitrogen) for 60 min. After another wash with TNBS for 3×5 min, the cells were counterstained with Hoechst 33342 at a concentration of 20 μg/ml for 20 min at room temperature. After a final wash with PBS, the stained cells were mounted by fluoroguard antifade reagent (Bio-Rad, Hercules, CA). At least 500 cells were counted using a fluorescent microscope (Zeiss Axioplan 2, Oberkochen, Germany).

MN scoring

The frequencies of MN were measured using the cytokinesis block technique (30). Briefly, cytochalasin B (Sigma) was added into the culture medium at a final concentration of 2.0 μg/ml and the cultures were incubated at 37°C. After 24 h, the cells were rinsed with PBS solution, fixed in a fixing solution (methanol: acetic acid = 9:1) for 20 min, stained with Hoechst 33342 (Sigma) at a concentration of 20 μg/ml for 20 min and viewed under a fluorescence microscope (Zeiss Axioplan 2) (30). At least 500 binucleate cells were examined and the yield of micronucleus formation (Y_{MN}) was calculated as $Y_{MN} = a/b$, where a was the total number of micronucleated cells scored and b was the total number of binucleate cells examined.

Measurement of O_2^- with hydroethidine staining

The production of O_2^- in the bystander cells was measured by assessing the fluorescence intensity of cells stained with hydroethidine (HE) (Invitrogen) (31). The production of O_2^- converts HE to ethidium bromide, resulting in an increase in red fluorescence. At 5 h post-cell mixing, the cell population was stained with 5 μM HE for 30 min at 37°C. After excessive dye was removed, the cells were rinsed with prewarmed D-hank's buffer solution twice, and the fluorescence images of at least 100 randomly selected cells per sample were captured using a fluorescent microscope (Zeiss Axioplan 2). The exposure conditions were the same in each experiment. The fluorescence intensity of bystander cells was measured and analyzed with the software Image J (http://rsb.info.nih.gov/ij/). To preclude the possible influence of other chemicals to HE oxidation signal, 100 U/ml superoxide dismutase–polyethylene glycol (Sigma) was used to treat the culture at 2 h before irradiation and then the fluorescence intensity of bystander cells was measured in some experiments.

Statistics

Data are presented as means ± SDs. Significance levels are assessed using Student's *t*-test. A *P*-value of 0.05 or less between groups is considered to indicate a statistically significant difference.

Results

Timing of BP1 formation in bystander population

The formation of BP1 in the bystander population was detected at chosen time points after cell mix. The results in Figure 1 showed that the fraction of BP1-positive cells in the bystander population increased after mixing with 10 cGy α-particle irradiated cells. At 5 h, the fraction of BP1-positive cells reached the maximum and then the yield decreased. Furthermore, the irradiated cells, which were subsequently seeded into the dish and mixed with the bystander cells, had adhered and stretched on the dish surface at 5 h after cell mixing. As such, 5 h was chosen as a representative time point in the subsequent experiments to study the protective effect of CO (CORM-2) on the bystander cells.

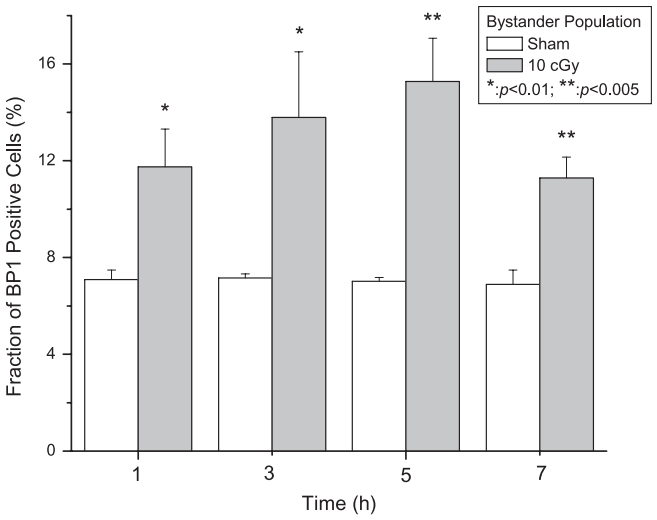

Fig. 1. The fraction of DSB (BP1)-positive cells in the bystander cell population at different time points after mixing with cells irradiated with 10 cGy α-particles. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and $P < 0.05$ is considered statistically significant.

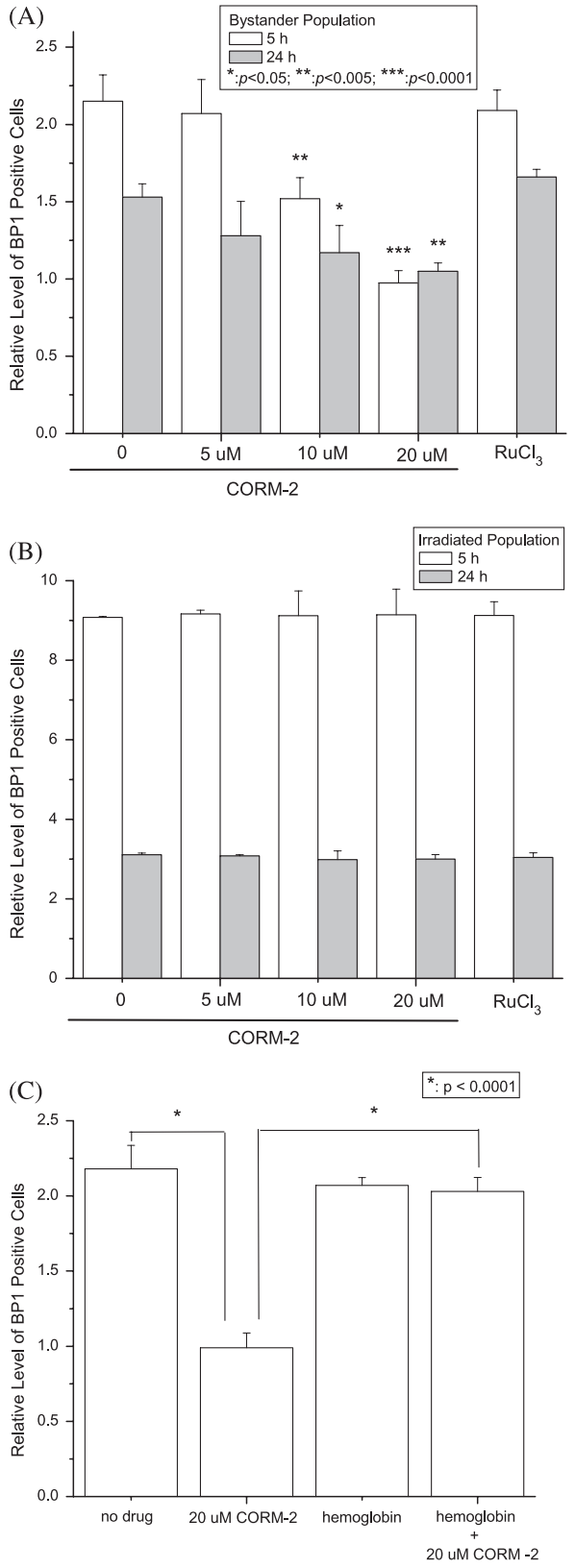

Fig. 2. Relative levels of BP1-positive cells in the irradiated (**B**) or non-irradiated bystander cell population (**A**) with the pretreatment of CORM-2 at 5 and 24 h post-irradiation. The background controls in each CORM-2 concentration were sham-irradiated samples treated with the same corresponding CORM-2 concentrations. The fractions of BP1-positive cells for individual treatment concentrations of CORM-2 are shown in supplementary Table 1 (available at *Carcinogenesis* Online). (**C**) Illustrations of the effect of hemoglobin treatment to the protective effect of CO released by 20 μM CORM-2. Data were pooled from three independent experiments. Significances in the differences between the sample and its control without CORM-2 treatment at the same time point are determined, and $P < 0.05$ is considered statistically significant.

CO (CORM-2) decreased DSB and MN formation in the bystander population

DSB (BP1) formation was measured at 5 or 24 h post-cell mixing. The time point of 24 h was chosen to assess the effect of CORM-2 on the formation of BP1 induced by RIBE after a relative long period. Results in Figure 2 showed the relative level of BP1-positive cells in the bystander or irradiated population with or without CO (CORM-2) treatment compared with the corresponding sham-irradiated control. RIBE also could be detected at 24 h with BP1 assessment, though the relative level of BP1 positive cells at 24 h was lower than that at 5 h due to the activation of DSB repair. These results indicated that in the bystander population the relative level of BP1-positive cells decreased in a manner dependent on the CORM-2 concentration at 5 h or 24 h after cell mixing. When the concentration was increased to 20 μM, the BP1 level was completely reduced to the background level. $RuCl_3$ (20 μM), which is a main product of CORM-2 except CO, in dimethyl sulfoxide was employed to treat cells in the control experiments and the results showed no distinct decrease in the BP1 levels. Furthermore, treatment with hemoglobin attenuated the protective effect of CO (CORM-2) distinctly and the BP1 level in bystander population was similar to the BP1 level without any drug treatment ($P = 0.209$) (Figure 2C). These results indicated that CO released by CORM-2 led to a reduction in the BP1 formation in the bystander cells. However, in the irradiated population, the treatment with CORM-2 did not decrease the level of BP1 significantly. Here, the formation of BP1 was produced by direct α-particle irradiation and CO (CORM-2) treatment might have no distinct protective effect on the irradiated cells. Treatment with only CORM-2 did not affect the level of BP1 significantly. The background level of BP1 for each CORM-2 concentration was shown in supplementary Table 1 (available at *Carcinogenesis* Online).

RIBE was also measured with MN assay, which indicated chromosome damage or aneuploid. The effect of CO (CORM-2) on MN formation in the irradiated or bystander populations (Figure 3) showed similar trends to the results with BP1 assessment. With the application of CO (CORM-2), the relative MN level decreased in the bystander population but not in the irradiated population. CORM-2 with a concentration of 20 μM led to almost complete attenuation of MN induced by RIBE.

NO or activation of NADPH mediate the bystander effect in the mixed coculture system

In a further study, the possible mechanism of bystander effect transduction in the mixed coculture system was investigated. A NO synthase inhibitor N^G-methyl-L-arginine (200 μM) (Sigma) (32) or a specific inhibitor of NADPH oxidase, apocynin (100 μM, Sigma) (33), was used to treat cells at the time of cell mixing or at 1 h before cell mixing and were present in the culture until the cells were fixed. The results in Figure 4 showed that the excessive BP1 formation in the bystander population was significantly attenuated to the level of sham-irradiated with N^G-methyl-L-arginine or apocynin treatment. This indicated that NO or $O_2^{\cdot-}$, which were produced by NO synthase or the activation of NADPH, respectively, mediated the transduction of bystander effect in the mixed coculture system.

CO (CORM-2) decreases the production of $O_2^{\cdot-}$ in bystander population

The production of $O_2^{\cdot-}$ was detected with HE. To preclude the influence of other oxidative chemicals produced in the bystander cells on the fluorescence signal, a specific $O_2^{\cdot-}$ scavenger, superoxide dismutase–polyethylene glycol, was used to treat the culture before irradiation. The results shown in supplementary Figure 1 (available at

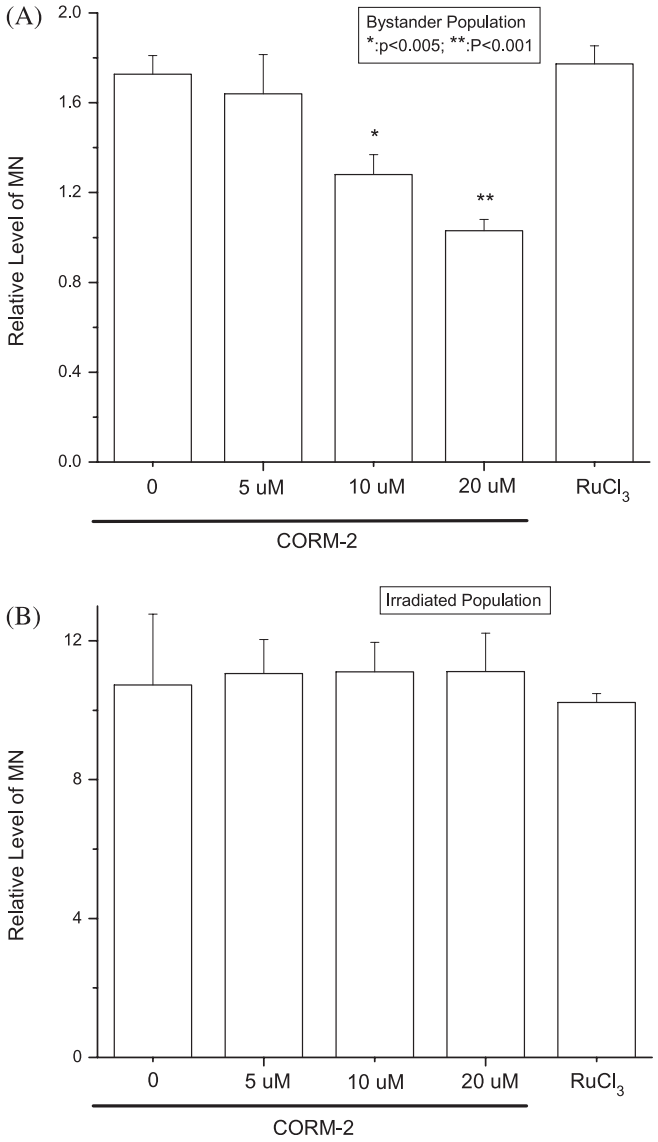

Fig. 3. Relative levels of MN yield in the irradiated (**B**) or non-irradiated (**A**) bystander cell population with the pretreatment of CORM-2. The background controls in each CORM-2 concentration were sham-irradiated samples treated with the same corresponding CORM-2 concentrations. Data were pooled from three independent experiments. Significances in the differences between the sample and its control without CORM-2 treatment are determined, and $P < 0.05$ is considered statistically significant.

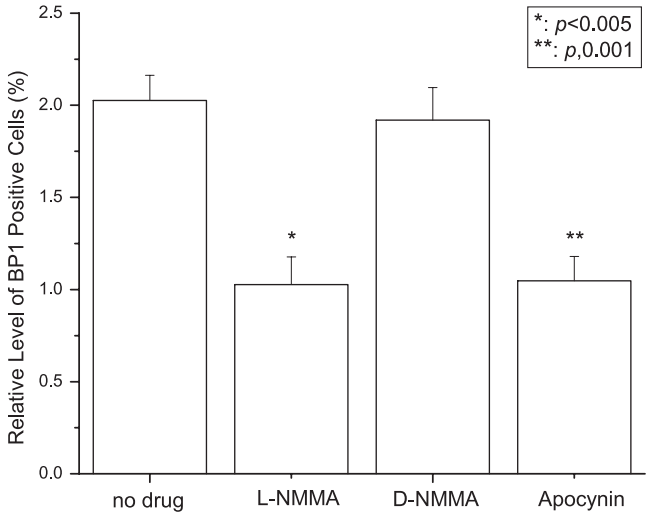

Fig. 4. The inhibitory effect of 200 μM N^G-methyl-L-arginine (L-NMMA) (a NO synthase inhibitor), 200 μM N^G-methyl-D-arginine (D-NMMA) (an inactive D-enantiomer) and 100 μM apocynin (a specific inhibitor of NADPH oxidase) on RIBE. The control was not treated with any drugs. Data are pooled from three independent experiments and the results represent mean ± SD. Significances in the differences between the samples are determined, and $P < 0.05$ is considered statistically significant.

Carcinogenesis Online) showed that pretreatment with superoxide dismutase–polyethylene glycol decreased the fluorescence intensity distinctly when compared with the case without any drug treatment. This result gave support to the production of $O_2^{\cdot-}$ in the bystander cells. The results in Figure 5 showed that $O_2^{\cdot-}$ could be induced significantly in the bystander population after mixed with irradiated cells when compared with the controls. The increased level of $O_2^{\cdot-}$ was an important mediator of bystander effect, and the produced $O_2^{\cdot-}$ would cause DNA or chromosome damages (31,34). With 20 μM CORM-2 treatment, the level of $O_2^{\cdot-}$ decreased to the background level. This indicated that CO (CORM-2) could attenuate the amount of $O_2^{\cdot-}$ in the bystander population.

CO (CORM-2) decreases the formation of DSB and MN induced by exogenous NO or H_2O_2

In a further experiment, sper (Sigma) was used as a NO generator to treat cells to mimic NO-mediated RIBE, and H_2O_2 (Sigma) was also used to generate $O_2^{\cdot-}$ to introduce ROS stress in the CHO cells to mimic the increased $O_2^{\cdot-}$ or ROS stress in the bystander cells. Sper or H_2O_2 was added into the culture and was present until the cells were fixed. The results in Figure 6 showed that sper or H_2O_2 could effectively induce formation of DSB (BP1) and MN in the CHO population. With 20 μM CORM-2 treatment, the relative level of MN and DSB decreased distinctly, and this protection effect was more significant when the cells were treated with lower concentrations of sper (0.75 μM) or H_2O_2 (10 μM). These results indicated that CO (CORM-2) could effectively protect CHO cells against the NO- or ROS-induced DNA or chromosomal damages.

Discussion

Studies on the mechanisms of RIBE revealed that ROS or NO acted as very important signal(s) in mediating the transduction of RIBE, and inflammatory-type response was also observed in RIBE (3,35). Low concentration of CO has been used therapeutically because of its anti-inflammatory and cytoprotective properties against ROS or NO stress (reviewed in ref. 10). In the present study, low-dose CO generated by CORM-2 led to a reduction in the formation of BP1 or MN in the bystander cells induced by RIBE, which were mediated by NO or ROS, and CO (CORM-2) could also protect the cells against the toxicity of NO generator sper or H_2O_2.

In previous studies on NO-mediated RIBE, excessive NO was directly measured in the bystander cells (8,36,37) or in the medium harvested from an AG 1522 cell population irradiated by low-dose α-particles (32) as a signaling molecule to mediate RIBE. The toxicity of NO is linked to its ability to combine with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$), an oxidizing free radical that can cause DNA fragmentation and lipid oxidation (38,39). In the present study, 20 μM CORM-2 could lead to a reduction in RIBE-induced BP1 and MN formation, and CORM-2 also protected cells against the toxicity of sper, which was used to generate NO to mimic the situation in bystander cells in the NO-mediated RIBE (40). These results showed a protective effect of CO (CORM-2) against reactive nitrogen species stress in bystander cells. CO has been reported to be able to protect cells from the cytotoxicity of NO or peoxynitrite. Li *et al.* reported that CO produced by HO-1 could rescue PC12 cells from nitrosative stress, which was produced by treating cells with NO generator

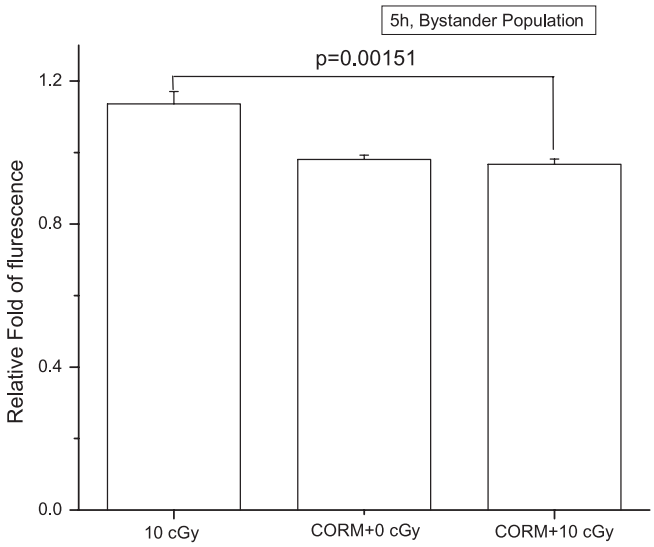

Fig. 5. Relative levels of $O_2^{\cdot-}$ fluorescence in the bystander population. The control was the sham-irradiated sample without chemical treatment. Data were pooled from three independent experiments. Significances in the differences between the sample and its respective control are determined, and $P < 0.05$ is considered statistically significant.

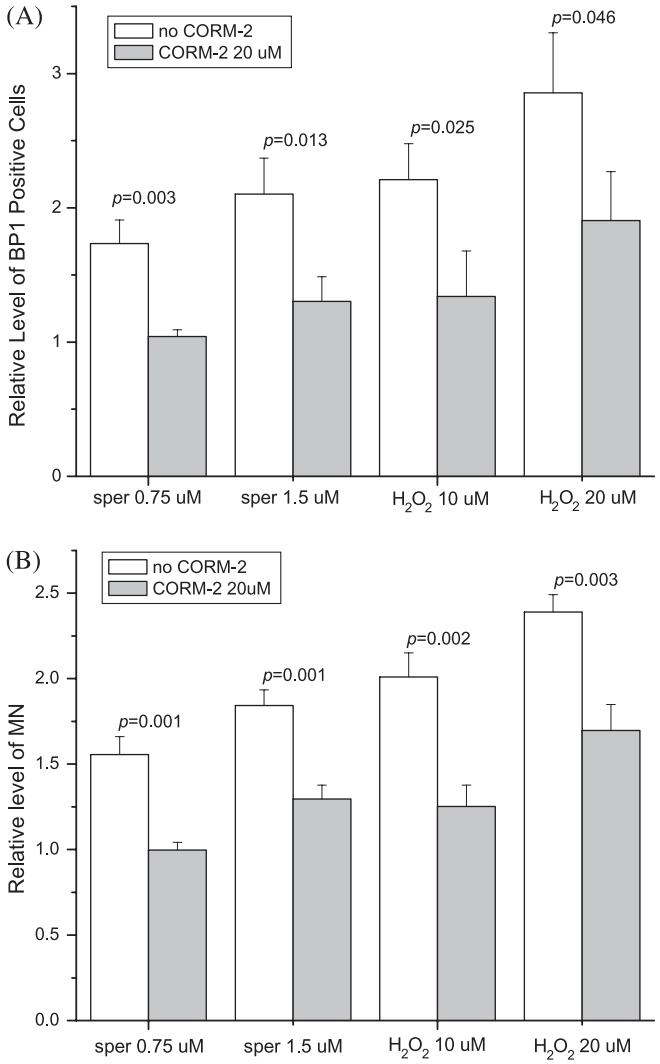

Fig. 6. The inhibitory effect of CORM-2 (20 μM) on the relative level of (**A**) BP1-positive cells and (**B**) MN with exogenous sper or H_2O_2 treatments. The controls were the samples without sper or H_2O_2 treatments. The *P*-value marked in the figure showed the significance between two adjacent columns, which represented samples with or without CORM-2 treatment. Data were pooled from three independent experiments. Significances in the differences between the sample and its respective control are determined, and $P < 0.05$ is considered statistically significant.

3-morpholinosydnonimine. CO induced an increase in glutamate–cysteine ligase, which is the rate-limiting enzyme in glutathione biosynthesis, and glutathione is an important antioxidant responsible for the maintenance of intracellular redox balance (41). CO could also protect PC12 cells from apoptotic death induced by peroxynitrite, generated by 3-morpholinosydnonimine, via preventing the depolarization of mitochondrial transmembrane potential (42). It was reported that in the inflamed tissue, the increased level of NO could be attenuated by treatment with CO (14). In addition, CO may also modulate the NO signaling to defend against the action of NO through reducing the expression of NO synthase or limiting the function of NO synthase (43,44), which is one of heme proteins, through the binding of CO to heme proteins to inhibit the function (reviewed in ref. 10).

The elevated ROS stress, especially that from $O_2^{\cdot-}$, was also measured in the bystander cells and it was demonstrated to participate in the DNA or chromosome damages in bystander cells (31,34). In the present study, the inhibitory effect of treatment with apocynin, a specific inhibitor of NADPH oxidase enzymes, and direct detection of $O_2^{\cdot-}$ with HE in bystander cells showed the involvement of NADPH oxidase activation in RIBE in the mixed coculture system, and this result agreed with those in former studies (45). It is known that NADPH oxidase produces $O_2^{\cdot-}$ in quantities capable of stimulating signaling pathways, and these enzymes are rapidly activated by a variety of soluble mediators and engagement of cell surface receptors (46). Attenuation of the increased level in of $O_2^{\cdot-}$ through treatment with CO (CORM-2) suggested that CO (CORM-2) had a potential inhibitory effect in decreasing the excessive production of $O_2^{\cdot-}$ in the bystander cells. It has been reported that CO can modulate the function of NADPH oxidase to attenuate the $O_2^{\cdot-}$ production since NADPH oxidases are a group of heme proteins and CO can bind to heme to inhibit its function (13). On the other hand, CO can upregulate the expression of glutamate–cysteine ligase to increase antioxidant glutathione to protect the cells (41). The result that CO (20 μM CORM-2) treatment protected cells against the cytotoxicity (DNA or chromosomal damage) of H_2O_2 demonstrated the antioxidative effect of CO, which agreed with the former studies.

CO might also affect the other reported RIBE signaling pathways such as the cyclooxygenase-2 (COX-2) signaling pathway (47). In this inflammatory-type pathway, binding of some cytokines, such as tumor necrosis factor-α, transforming growth factor-β, interleukin-1 etc., to the corresponding receptor can activate signaling pathways, including IκB kinase-mediated phosphorylation of IκB, which releases NF-κB to enter the nucleus that acts as a transcription factor for COX-2 and inducible NO synthase, and mitogen-activated protein kinase pathways (extracellular signal-regulated kinase, c-jun N-terminal kinase and p38) to upregulate the expression of COX-2. Activation of COX-2 provides a continuous supply of reactive radicals and cytokines for the propagation of the bystander signals (22,37,48). Some recent reports have demonstrated the function of CO on anti-inflammation. CO can inhibit the production of pro-inflammatory cytokines, such as tumor necrosis factor-α, migration inhibitory factor and interleukin-1, -2 and -10 through p38 mitogen-activated protein kinase-dependent pathways, leading to anti-inflammatory tissue protection (10,49). In addition, activation of NF-κB, which is a very important transcription factor in COX-2 signaling pathway, can be attenuated by treatment with CO in the inflammation tissue (14). Therefore, in the transduction of bystander effect, CO might modulate or affect these signaling pathways through attenuation of NF-κB activation, production of cytokines or modulation of the mitogen-activated protein kinase pathway. The ascertainment on whether CO

can actually modulate these signaling pathways in RIBE will need more evidences from further studies.

The protective effect of CO (CORM-2) against RIBE and the wide use of CO in the treatment of inflammatory diseases, lung injuries, cardiovascular injuries etc., by HO gene transfer, inhalation of CO or using CORMs (reviewed in refs 10,11) have hinted the potential use of CO in protecting the normal tissues during radiotherapy.

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