Transcutaneous application of CO_2 enhances the antitumor effect of radiation therapy in human malignant fibrous histiocytoma

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Abstract. Sarcomas are relatively resistant because of hypoxia. We previously demonstrated that the transcutaneous CO_2 therapy reduced hypoxic conditions in human malignant fibrous histiocytoma (MFH). Therefore, we hypothesized that transcutaneous CO_2 therapy could enhance the antitumor effect of radiation therapy in human MFH. Our purpose was to evaluate the effects of transcutaneous CO_2 therapy on the antitumor efficacy of X-ray irradiation using MFH. First, in an *in vitro* study, we assessed apoptotic activity and reactive oxygen species (ROS) production using flow cytometric and immunoblot analysis at 24 h after X-ray irradiation under three different oxygen conditions (normoxic, reoxygenated and hypoxic). In addition, in the *in vivo* study, 24 male athymic BALB/c nude mice with MFH tumors that were inoculated in the dorsal subcutaneous area were randomized into four groups: control, CO_2, X-ray irradiation and combination (CO_2 and X-ray irradiation). Treatments were performed twice weekly for 2 weeks, four times in total. Tumor volume was calculated. All tumors were excised and apoptotic activity, ROS production, related proteins and HIF-1α expression were assessed using flow cytometric and immunoblot analysis. The *in vitro* study revealed that X-ray irradiation induced increased apoptosis and ROS production in MFH cells under normoxic and reoxygenated conditions relative to hypoxic conditions (P<0.01). In the *in vivo* study, tumor volume in the combination group was reduced to 28, 42 and 47% of that in the control, CO_2, and X-ray groups, respectively (P<0.05). Apoptotic activity and ROS production in the combination group were strongly increased with decreasing HIF-1α expression relative to the control, CO_2 and X-ray groups. The transcutaneous CO_2 system enhanced the antitumor action of X-ray irradiation and could be a novel therapeutic tool for overcoming radioresistance in human malignancies.

Introduction

Malignant fibrous histiocytoma (MFH), which has recently been classified as undifferentiated pleomorphic sarcoma (UPS), is the most common soft tissue sarcoma in late adult life. Advances in the treatment of MFH have led to multi-disciplinary treatments, including surgery, chemotherapy and radiation therapy. However, the prognosis of patients with the disease is still extremely poor because of local recurrence and distant metastases (1).

Tumor hypoxia is a common feature of malignant tumors, and contributes to their malignant progression, distant metastasis and resistance to radiation therapy and chemotherapy (2). The relevance of tumor oxygenation to radiocurability was first proposed by Gray *et al* (3). Since the role played by oxygenation in radiosensitivity has been recognized, several strategies including high-oxygen-content gas breathing, radio-sensitizers, and hypoxic cytotoxins, have been developed to overcome hypoxia-mediated radioresistance (4). However, with these strategies an increased tumor control rate is often accompanied by more severe side effects (5,6).

Carbon dioxide (CO_2) therapy in the form of a carbonated spa has been historically used in Europe as an effective treatment for cardiac diseases and skin problems (7,8). It has been suggested that the therapeutic effects of CO_2 may be due to an increase in blood flow and microcirculation, nitric oxide-dependent neocapillary formation, and a partial increase in O_2 pressure in the local tissues known as the Bohr effect (7-9). We have previously demonstrated that the transcutaneous application of CO_2 increased local O_2 pressure in treated tissues, potentially causing an 'artificial Bohr effect' (10), and we have also shown that the transcutaneous CO_2 system has antitumor effects; it can also enhance chemosensitivity through the improvement of hypoxia in human MFH xenografts (11,12). In murine osteosarcoma cells, the transcutaneous CO_2 system has also been reported to reduce hypoxic conditions (13). Based on these findings, we

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hypothesized that the transcutaneous application of CO_2 could enhance the antitumor effects of radiation therapy through the improvement of hypoxia in tumor tissues.

In the presence of oxygen, ionizing radiation generates reactive oxygen species (ROS) such as superoxide radicles, hydrogen peroxide and hydroxyl radicals; these show high reactivity to a variety of cellular macromolecules (14). ROS are implicated in playing crucial roles in cellular responses to radiation therapy (14), and they also regulate a broad array of signal transductions that control various biological processes including apoptosis (15). Mitogen-activated protein kinases (MAPKs) include three major kinases: extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). Among these kinases, p38 and JNK/SAPK are mainly activated by extracellular stresses such as irradiation and inflammatory cytokines (16). Recent studies have shown that activation of p38 and JNK/SAPK contributes to ROS-induced apoptosis (17,18). Hypoxia is thought to induce apoptotic resistance by suppression of caspase-3 and JNK/SAPK in cells (17).

Additionally, it is well documented that ROS are involved in the induction of apoptosis in γ-irradiated cancer cells as well as normal cells (18). However, the effects of hypoxia on radiation induced apoptotic activities in MFH cells are poorly understood, and the relationship between ROS production and the induction of apoptosis by radiation therapy in MFH has not been reported. In the current study, we examined the *in vitro* effects of altering the oxygen conditions on X-ray irradiation induced cell apoptosis and ROS production, and the activation of ROS-mediated and apoptosis related proteins in MFH cells. We also utilized a murine model of human MFH to examine the antitumor effects of combined treatment with CO_2 therapy and X-ray irradiation. Additionally, we investigated the effects of transcutaneous CO_2 application on radiation therapy in terms of ROS production, the induction of apoptosis, and the activation of ROS-mediated and apoptosis- related proteins in human MFH.

Materials and methods

Cell cultures. Four human MFH cell lines (Nara-H, TNMY-1, Nara-F and GBS-1) were used in our studies. Nara-H and Nara-F cell lines were obtained from ScienStuff Co. (Nara, Japan) (19). TNMY-1 was previously established in our laboratory (20). GBS-1 was obtained from ACTT (21). Cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM: Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS: Sigma-Aldrich Co.) and 100 U/ml penicillin/streptomycin solution (Sigma-Aldrich Co.). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

X-ray irradiation. X-ray irradiation was performed at a dose rate of 0.64-0.66 Gy/min using a 150 kV X-ray generator unit operating at 5 mA with an external 0.1 mm aluminum filter (MBR-1505122: Hitachi Medical Co., Tokyo, Japan).

Colony formation assay. We performed colony formation assays of X-ray irradiated MFH cells to evaluate the response of the cells to X-ray irradiation. Four MFH cell lines were
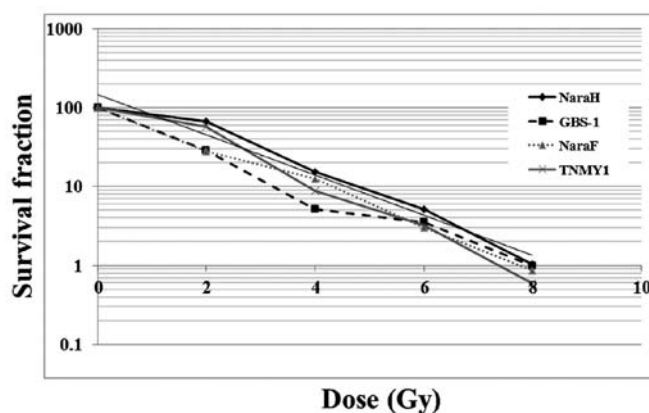

Figure 1. Effect of X-ray irradiation on the survival of human MFH cell lines (Nara-H, Nara-F, TNMY-1, and GBS-1) *in vitro*.

treated by X-irradiation at five different doses (0, 2, 4, 6 and 8 Gy). Immediately after X-irradiation, cells were trypsinized and seeded at a density of 100-1000 cells/well in 6-well plates, and incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 2 weeks of incubation, cells were stained with Giemsa and the number of colonies was counted.

In vitro experiments. To investigate the effects of altering the oxygen conditions on the sensitivity of human MFH cells to X-ray irradiation, Nara-H cells were incubated for 48 h under one of three different oxygen conditions: normoxic (20% O_2, 5% CO_2, and 75% N_2), hypoxic (1% O_2, 5% CO_2, and 94% N_2), or reoxygenated conditions, as previously described (12). Under reoxygenated conditions, cells were incubated under normoxia for 24 h followed by 24 h of incubation under hypoxic conditions, as previously described (12). Hypoxic conditions were also obtained by exposing Nara-H cells to $CoCl_2$, a well known chemical used to induce of hypoxia (23). In particular, the $CoCl_2$ powder was dissolved in DMEM and the resulting solution was filtered and then added to cell cultures at final concentration of 150 μl (23). In the reoxygenated condition, the medium without $CoCl_2$ (normoxic condition) was replaced after 24 h under $CoCl_2$ hypoxic conditions. We also examined the effects of altering the oxygen conditions on the sensitivity of human MFH cells to X-ray irradiation. After 24 h of incubation in one of three different oxygen conditions X-ray irradiation (3.2 Gy) was performed, and incubation was continued for a further 24 h.

Animal models. Male athymic BALB/c nude mice, aged 5-8 weeks, were obtained from CLEA Japan Inc. (Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Kobe University Graduate School of Medicine, and were performed in accordance with the Guidelines for Animal Experimentation of Kobe University Graduate School of Medicine, and Kobe University Animal Experimentation Regulations (permission number: P110201). To create a murine model of human MFH, Nara-H cells were implanted into the dorsal subcutaneous area of male athymic BALB/c nude mice at a concentration of 4.0×10^6 cells in 500 μl PBS as previously described (11). Transcutaneous application of
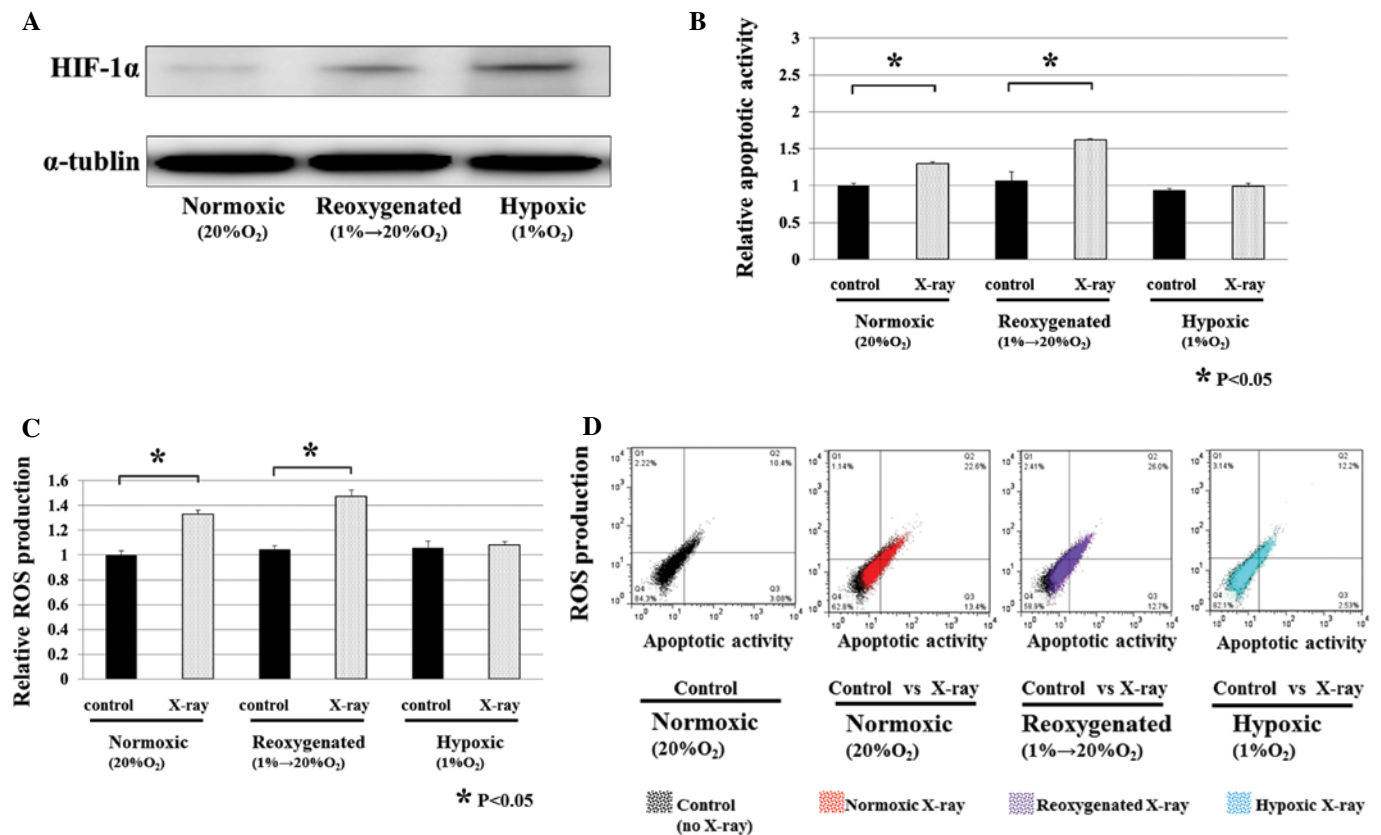
Figure 2. Effect of altering oxygen conditions on X-ray induced apoptotic activity and ROS production in human MFH cells *in vitro*. (A) Immunoblot analysis of HIF-1α expression under three different oxygen conditions. (B) Apoptotic activity and (C) ROS production were evaluated in human MFH cells which were treated with X-rays (3.2 Gy) under three different oxygen conditions. Results were normalized to the mean value of the normoxic control. Data represent the mean ± SE of at least three independent experiments ($^*P<0.05$). (D) Correlation between apoptotic activity and ROS production in X-ray irradiated MFH cells under three different oxygen conditions (black, normoxic control; red, normoxic X-rays; purple, reoxygenated X-rays; blue, hypoxic X-rays).

CO_2 was carried out as previously described (11,12). Each treatment was performed for 10 min. Control animals were treated in a similar manner by replacing CO_2 with room air.

In vivo MFH tumor studies. We examined the *in vivo* effects of combined treatments using transcutaneous CO_2 therapy and X-ray irradiation. Mice were randomly divided into four groups: the control group (n=6), the CO_2 group (n=6), the X-ray irradiation group (n=6), and the combination (CO_2 and X-ray irradiation) group (n=6). Treatments commenced 3 days after MFH cell implantation, and were performed twice weekly for 2 weeks, four times in total. X-ray irradiation was given immediately after CO_2 or room air treatment at a dose of 0.8 Gy for each treatment (3.2 Gy in total). Mouse body weight and tumor volume were monitored twice weekly until the end of treatment. Tumor volume (V) was calculated as previously described (11,12). All tumors were excised at the end of treatment and apoptotic activity, ROS production and their relationship in tumor tissue was assessed.

Flow cytometric analysis. Flow cytometry was performed using a FACS Calibur™ flow cytometer (BD Pharmingen, Franklin Lakes, NJ, USA) as previously described (11,12). We evaluated apoptotic activity in MFH cells by means of the DNA fragmentation assay using the Apo-Direct kit according to the manufacturer's protocol (BD Pharmingen). To evaluate ROS production we used 2', 7'-dichlorofluorescin diacetate

(DCFH-DA; Molecular Probes, Invitrogen, Carlsbad, CA, USA) as an indicator (22).

Immunoblot analysis. Immunoblot analysis was performed as previously described (11,12). We used the following antibodies: anti-human caspase-3 antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA), anti-human PARP antibody (1:1000) (Cell Signaling Technology), anti-human p38 antibody (1:1000) (Cell Signaling Technology), anti-human phospho p38 antibody (1:1000) (Cell Signaling Technology), anti-human JNK/SAPK antibody (1:1000) (Cell Signaling Technology), anti-human phospho-JNK/SAPK antibody (1:1000) (Cell Signaling Technology), and anti-human α-tubulin antibody (1:2000) (Sigma-Aldrich Co.).

Statistical analysis. All experiments were performed independently at least three times, and data are presented as the mean ± standard error (SE) unless otherwise indicated. Differences between groups were evaluated using a two-tailed Student's t-test, and also using ANOVA with the post hoc test to compare for continuous values. All tests were considered significant when the P-value was <0.05.

Results

Effects of oxygen conditions on X-ray induced apoptosis and ROS production in human MFH cells in vitro. First, we
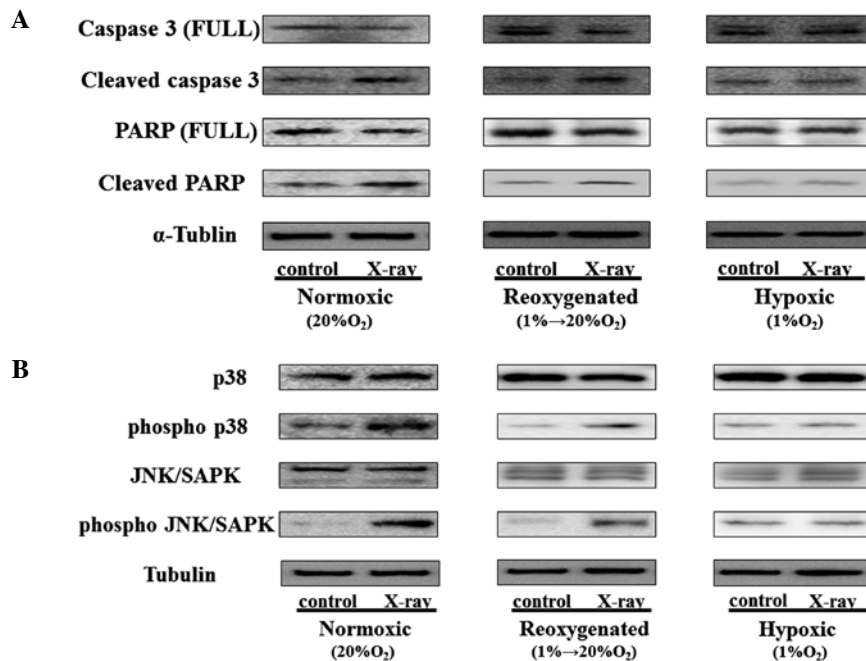
Figure 3. *In vitro* effects of X-ray irradiation on the expression of apoptosis related proteins and ROS-mediated signaling pathways under three different oxygen conditions in human MFH cells. (A) The full length and cleavage of apoptosis related proteins, caspase-3 and PARP in MFH cells. (B) Activation of the ROS-mediated signaling pathways, p38 and JNK/SAPK in X-ray irradiated MFH cells under three oxygen conditions.

performed colony formation assays using four MFH cell lines (Nara-H, Nara-F, TNMY-1, and GBS-1) that were treated with various doses of X-rays to evaluate the response of the cells to radiation therapy. The number of MFH cell colonies decreased in a dose-dependent manner after X-ray irradiation. Nara-H cells were the most radioresistant of the four MFH cell lines; a 50% decrease in colony formation was achieved at a dose of 3.2 Gy (Fig. 1).

Next, we studied the effects of altering the oxygen conditions on X-ray induced cell apoptosis in Nara-H cells *in vitro*. Three different oxygen conditions were used as previously described (12). Apoptotic activity and ROS production in the cells was assessed using flow cytometric analysis and immunoblotting at 24 h after X-ray irradiation (3.2 Gy) under three different oxygen conditions. After 48 h of incubation, the expression of HIF-1α protein was strongly increased in cells under hypoxic conditions, whereas the expression of HIF-1α protein was decreased in cells cultured under reoxygenated conditions (Fig. 2A). It was found that both apoptotic activity and ROS production were increased by X-ray irradiation in reoxygenated and normoxic conditions ($P<0.05$) (Fig. 2B and C). In addition, there was a correlation between apoptotic activity and ROS production (Fig. 2D). However, neither the apoptotic activity or ROS production was increased by X-ray irradiation under hypoxic conditions (Fig. 2B and C). Hypoxic conditions were also stimulated by exposure to CoCl$_2$, a well known chemical used to induce hypoxia (23). These results were consistent with those obtained after the chemical induction (CoCl$_2$) of three different oxygen conditions (data not shown).

Immunoblot analyses revealed that the cleavage of both caspase-3 and PARP was strongly increased in X-ray irradiated MFH cells under normoxic and reoxygenated conditions (Fig. 3A). It also revealed that the expression of phosphorylated

forms of both of the pro-apoptotic signaling molecules, p38 and JNK/SAPK, were increased after X-ray irradiation, while the expression of total p38 and JNK/SAPK were not changed under normoxic and reoxygenated conditions (Fig. 3B); however, under hypoxic conditions the expression of protein was not changed (Fig. 3A and B). These results were corroborated by chemical induction of three different oxygen conditions, achieved by exposure to CoCl$_2$ (data not shown). They strongly indicated that the improvement in hypoxic conditions enhanced X-ray irradiation-induced MFH cell apoptosis and ROS production *in vitro*.

Effects of the transcutaneous application of CO$_2$ with X-ray irradiation on apoptosis and ROS production in human MFH xenografts. To examine the effect of transcutaneous CO$_2$ therapy on X-ray irradiation *in vivo*, we used the Nara-H cell line; the response of MFH tumors to this combined treatment was evaluated. We observed a significant decrease in tumor volume in the CO$_2$, X-ray irradiation and combination groups when compared with the control group. At the end of the experiment, tumor volume in the combination group (CO$_2$ and X-ray irradiation) was reduced to 28, 42 and 47% of that in the control, CO$_2$ alone, and X-ray irradiation alone groups, respectively (Fig. 4A). No significant differences in body weight were observed among the groups (Fig. 4B). FACS analyses showed that apoptotic activity and ROS production in the combination group were strongly increased relative to those in the control, CO$_2$ alone, and X-ray irradiation alone groups ($P<0.01$) (Fig. 5A and B). Transcutaneous CO$_2$ therapy alone did not affect ROS production (Fig. 5B). There was a correlation between apoptotic activity and ROS production in the X-ray irradiation alone and combination groups (Fig. 5C). In addition, we evaluated the effect of combined treatment with transcutaneous CO$_2$ therapy and X-ray irradiation on
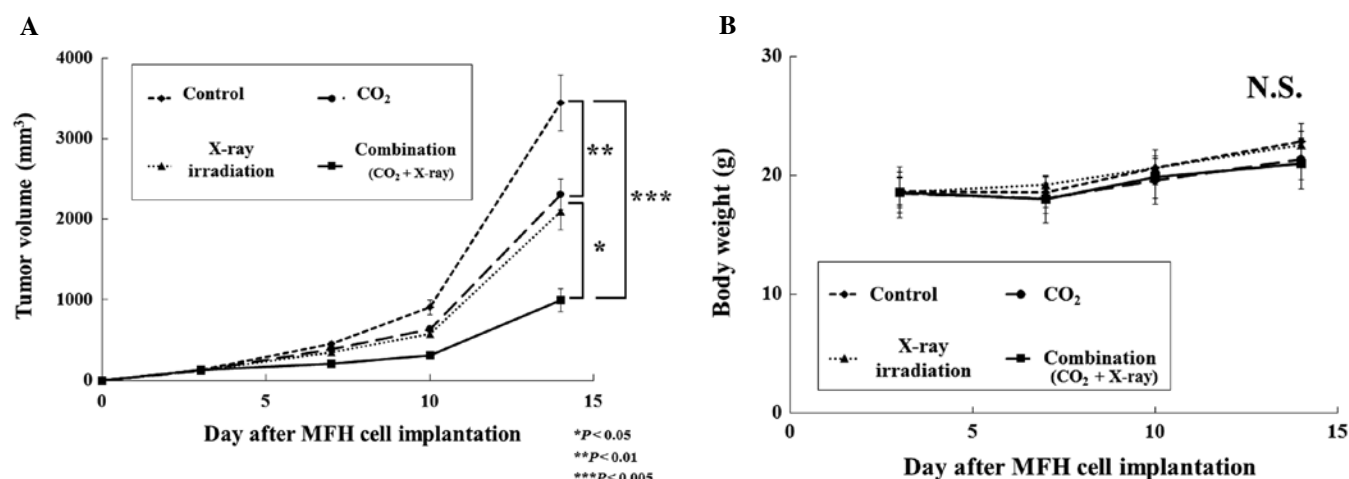
Figure 4. *In vivo* MFH cell growth after multiple treatments with transcutaneous CO_2 therapy and X-ray irradiation. (A) Tumor volume and (B) body weight in mice were monitored until the end of the experiment. Data represent the mean ± SE of at least three independent experiments (*P<0.05; **P<0.01; and ***P<0.005).
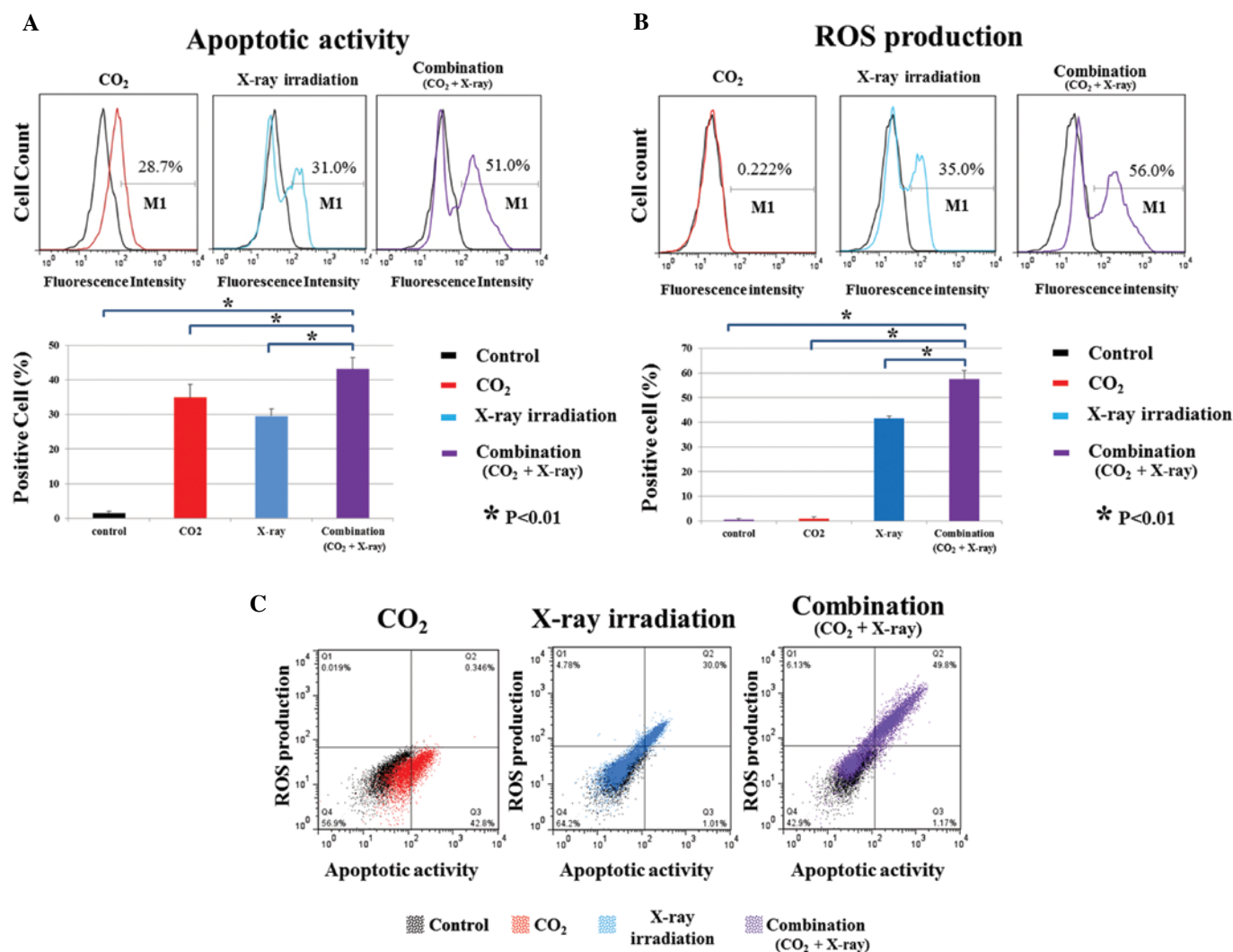

Figure 5. Effects of multiple treatments with transcutaneous CO_2 therapy and X-ray irradiation on apoptotic activity and ROS production in human MFH xenografts. (A) Apoptotic activity and (B) ROS production in tumors were evaluated using flow cytometric analysis (black, control group; red, CO_2 group; blue, X-ray irradiation group; purple, combination group). The cell population was measured in the M1 area. Data represent the mean ± SE of at least three independent experiments (*P<0.01). (C) Correlations between apoptotic activity and ROS production in the four groups.

the ROS-mediated signaling pathway. Immunoblot analyses showed that the expressions of the cleavage of caspase-3, and PARP and phosphorylated forms of both p38 and JNK/SAPK, were strongly increased with decreasing HIF-1α expression in
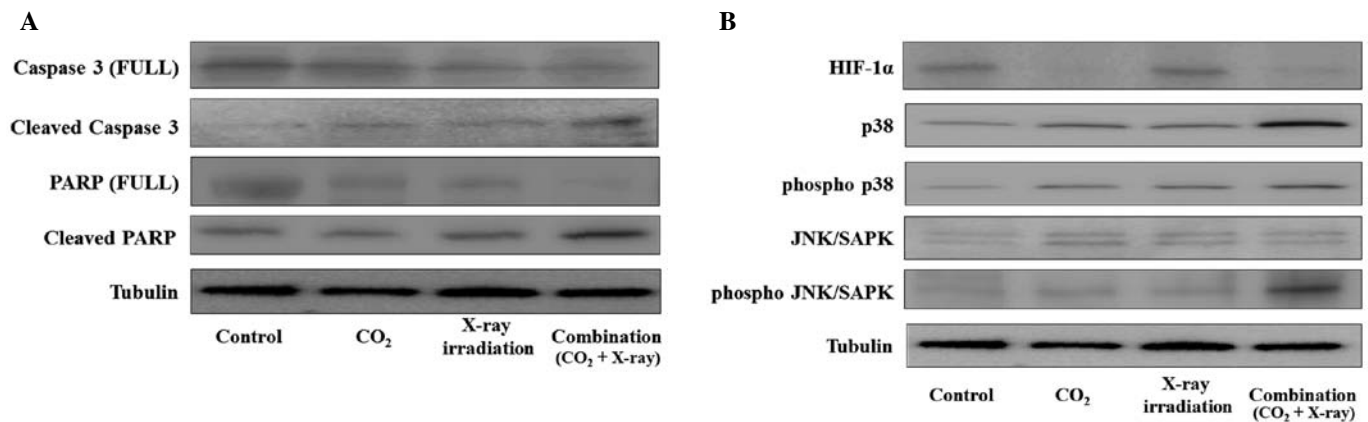
Figure 6. Effects of a single treatment with transcutaneous CO$_2$ therapy and X-ray irradiation on the ROS-mediated signaling pathway in human MFH *in vivo*. Immunoblot analyses for the expression of full length and cleaved caspase-3 and PARP (A), HIF-1α and the phosphorylated forms of p38 and JNK/SAPK (B) in treated MFH tumors.

tumors that were treated by combined treatment relative to the other treatment groups (Fig. 6). These results indicated that combined treatment with transcutaneous CO$_2$ therapy and X-ray irradiation resulted in a significant antitumor effect in the human MFH xenografts; in addition, transcutaneous CO$_2$ therapy enhanced the antitumor effect of X-ray irradiation with an improvement in hypoxic conditions through the activation of the ROS-mediated signaling pathway.

Discussion

The presence of hypoxic cells in solid tumors has been well documented, and tumor hypoxia is believed to be a major cause of clinical radioresistance in various cancer types including MFH (24-26). Previous studies dating back more than 50 years have shown that well oxygenated cells are three times more radiosensitive than hypoxic cells (3, 27). Based on these strategies, several studies have been performed using hyperbaric oxygen (28) and the transfusion of red blood cells (29) to radiosensitize tumor tissues. However, these therapies have proven to be of limited benefit and can be harmful to patients (5,6).

We have previously demonstrated in human MFH xenografts that the transcutaneous application of CO$_2$ increased the local O$_2$ pressure in treated tissue (10), and that CO$_2$ therapy enhanced chemosensitivity because of a reduction in hypoxia (11). We have also previously reported that CO$_2$ therapy decreased the metastatic potential of murine osteosarcoma (13). Therefore, we hypothesize that our transcutaneous CO$_2$ therapy can improve the response of MFH cells to radiation therapy. There is little evidence to support a possible relationship between CO$_2$ therapy and radiosensitivity. Carbogen, a hyperoxic gas containing a small fraction of CO$_2$, has been reported as being a more effective and less toxic radiosensitizer (30). Carbogen was first used clinically as a radiosensitizer in the early 1960s, and has now been reintroduced into the clinic following the work of Rojas *et al* (30). These authors demonstrated the effectiveness of simple normobaric oxygen and carbogen breathing in an animal tumor model (30). The gas has been shown to improve tumor oxygenation by increasing the amount of dissolved oxygen in plasma, and to increase tumor radiosensitivity (31).

In the current study, we demonstrated that the transcutaneous application of CO$_2$ enhanced the antitumor effects of X-ray irradiation in human MFH xenografts, with no observable side effects.

In addition, we found that a possible mechanism involved in the induction of MFH cell death after combined treatment with transcutaneous CO$_2$ therapy and X-ray irradiation, may depend on ROS production and the activation of ROS-mediated signaling pathways. In the presence of oxygen, ionizing radiation acts on biochemical systems via ROS production (32). ROS play a major role in radiation-induced cellular damage, and can induce apoptosis through activation of a pro-apoptosis pathway (33). In the current *in vivo* study, increased apoptotic activity along with increased ROS production were observed in the combination group (CO$_2$ and X-ray irradiation), while ROS production was not increased by transcutaneous CO$_2$ therapy alone. The results suggest that transcutaneous CO$_2$ therapy can enhance radiation-induced ROS production, but cannot increase ROS production by itself. Our previous study revealed that transcutaneous CO$_2$ therapy improved hypoxic conditions in MFH tumor tissues (11). Therefore, we speculate that transcutaneous CO$_2$ therapy can enhance ROS production in X-ray irradiated MFH tumors through increased local oxygenation. Pro-apoptotic signaling pathways, such as p38 MAPK and JNK/SAPK, are stimulated by ROS production and induce cell apoptosis (34,35). It has been reported that p38 MAPK was efficiently activated by a low dose of X-rays (36), and radiation-induced upregulation of MAPKs and JNK/SAPK has also been reported in cultured cells (37,38). However, radiation-induced upregulation of MAPKs or JNK/SAPK in musculoskeletal tumors has not been studied. In the present study, we found that p38 and JNK/SAPK were activated in human MFH tumors after X-ray irradiation in reoxygenated conditions *in vitro*; in addition, the activation of both p38 and JNK/SAPK were strongly increased with the improvement in hypoxic conditions in xenograft bearing mice that were treated using combined treatment with transcutaneous CO$_2$ therapy and X-ray irradiation, relative to CO$_2$ therapy alone or X-ray irradiation alone. These results suggest that the mechanism of enhancement of the antitumor effects of radiation therapy by transcutaneous CO$_2$ therapy involves ROS production and

ROS-mediated activation of p38 and JNK/SAPK signaling pathways. However, the ROS-related mehcanisms require further study.

In conclusion, the findings of the current study strongly imply that CO_2 therapy can enhance the antitumor effect of radiation therapy for human MFH by increasing ROS production as a consequence of reduced tumor hypoxia, with no observable side effects. We believe that the current study is the first to reveal the effect of transcutaneous CO_2 therapy on the radiocurability of a human sarcoma xenograft tumor, and could represent a potent therapeutic breakthrough in the treatment of radioresistant human malignancies.

Acknowledgements

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