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Transcutaneous Application of Carbon Dioxide (CO_2) Enhances Chemosensitivity by Reducing Hypoxic Conditions in Human Malignant Fibrous Histiocytoma

Yasuo Onishi[1], Teruya Kawamoto[1]*, Takeshi Ueha[2], Hitomi Hara[1], Naomasa Fukase[1], Mitsunori Toda[1], Risa Harada[1], Yoshitada Sakai[3], Masahiko Miwa[1], Kotaro Nishida[1], Ryosuke Kuroda[1], Masahiro Kurosaka[1] and Toshihiro Akisue[1]

[1]*Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*
[2]*NeoChemir Inc, Sannomiya Chuo-building 4F, 4-2-20 Gokodori, Chuo-ku, Kobe 651-0087, Japan*
[3]*Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*

Abstract

Background: Tumor hypoxia is a common feature of various human malignancies. Hypoxia contributes to tumor progression, and is a major cause of tumor resistance to chemotherapy. Hypoxia-inducible factor (HIF)-1 is a key transcription factor in hypoxic responses, and regulates the transcription of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, and invasion. We previously demonstrated that transcutaneous application of carbon dioxide (CO_2) induced oxygenation in the treated tissue *in vivo*, therefore, we hypothesized that transcutaneous CO_2 exposure could enhance the chemosensitivity by reducing hypoxia in a tumor tissue. The aim of this study was to examine the effect of oxygenation by transcutaneous application of CO_2 on the therapeutic efficacy of doxorubicin (DOX) to treat human malignant fibrous histiocytoma (MFH) *in vivo*.

Methods: In this study, we utilized a murine model of human MFH, and mice were randomly divided into four groups: control, CO_2, DOX and combination (CO_2 + DOX) treatment groups to examine the effect of transcutaneous application of CO_2 on the hypoxic condition, and to assess the therapeutic effect of combination therapy using transcutaneous CO_2 and DOX treatment *in vivo*.

Results: Transcutaneous application of CO_2 treatment decreased HIF-1α expression in human MFH tumor tissues, suggesting that our transcutaneous CO_2 treatment reduced the hypoxic conditions. Furthermore, transcutaneous CO_2 treatment alone had an antitumoral effect, and increased the chemotherapeutic effect of DOX on MFH tumor growth *in vivo*, with no observable effects on body weight.

Conclusions: Our findings in this study strongly indicate that our transcutaneous CO_2 system has antitumor effects and can enhance the chemosensitivity of tumor cells by reducing the local hypoxic conditions.

Keywords: Transcutaneous application; Carbon dioxide (CO_2); Hypoxia; HIF-1α; Chemosensitivity; Malignant fibrous histiocytoma (MFH)

Introduction

Musculoskeletal malignancies, particularly high-grade sarcomas such as malignant fibrous histiocytoma (MFH), are clinically aggressive and demonstrate high metastatic behavior in various organs. Surgical excision is the primary mode of therapy for MFH patients; however, early consideration should be given to the use of adjuvant radiation therapy and/or chemotherapy due to the high recurrence and metastatic rate of the tumor [1]. Chemotherapeutic effects may be important in the survival of patients with the disease, and this systemic modality should be the treatment of choice for patients at an advanced stage [2]. Although many chemotherapeutic protocols are used to treat human sarcomas, the current chemotherapeutic strategies for high-grade sarcomas are ineffective and the prognosis of patients can be extremely poor due to local recurrence and metastases [1]. Among the approved anticancer drugs, doxorubicin (DOX) is one of the most widely used agents to treat sarcomas. However DOX has been reported to only have a 15% response rate in soft tissue sarcomas [3]. In the last 20 years, various cooperative groups have investigated combinations of different drugs as a first-line chemotherapeutic protocol for high-grade sarcomas in randomized trials [4]. Despite the higher response rate achieved in several studies, no multidrug regimen has demonstrated any advantage in terms of overall survival when compared with the single-agent, DOX [4]. However, there is evidence for a dose-response side effect of DOX treatment for soft tissue sarcoma. Hematologic toxicity, which is the most frequent side effect observed when a combination of the two most active drugs, (DOX and ifosfamide) are used, precludes any dose escalation of DOX within the optimal dose range [4]. Therefore, new strategies to enhance the therapeutic effect of DOX to treat high-grade sarcomas are needed.

A hypoxic microenvironment is a common feature of solid tumors, and induces various molecular pathways to allow tumor cells to become resistant to radiotherapy and chemotherapy [5,6]. Smith et al. [7], reported that chronically hypoxic cells in tumors were resistant to the cytotoxic action of DOX. In addition, exponentially growing cells in a culture which is rendered chronically hypoxic show more resistance to bleomycin [8], 5-fluorouracil, cytosine arabinoside and vincristine [9] than normally proliferating cells. Hypoxia-inducible factor-1 (HIF-1),

***Corresponding author:** Teruya Kawamoto, M.D, Ph.D, Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan, Tel: +81-78-382-5985; Fax: +81-78-351-6944; E-mail: trykwmt@med.kobe-u.ac.jp

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an oxygen-dependent transcription factor, mediates the adaptation of cancer cells to the hypoxic environment by controlling the expression of hundreds of genes [10,11], including vascular endothelial growth factor (VEGF) [12], glycolytic enzymes, and glucose transporters [13]. In addition, several studies have observed a correlation between HIF-1 expression and tumor progression [14-17]. HIF-1-deficient hepatoma cells [14] and lung cancer cells treated with siRNA targeting HIF-1α [15] are more susceptible to radiotherapy and chemotherapy, respectively, than parental and untreated cells. In musculoskeletal tumors, Yang et al. [16], observed that HIF-1 expression significantly affected the overall and disease-free survival in patients with osteosarcoma. Koga et al. [17], reported that the expression level of HIF-1α correlated with the microvessel density in MFH tissues, and that HIF-1α expression contributed to the malignant potential of MFH. Taken together, these reports suggest that tumor hypoxia plays a significant role in tumor progression, and that modification of tumor hypoxia can be considered an attractive therapeutic target in patients with sarcomas. Although strategies designed to improve tumor oxygenation have been investigated, no method has been widely accepted into clinical practice, although there is an evidence for a small improvement in outcome [18,19].

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 troubles [20,21]. The therapeutic effects of CO_2 are due to an increase of blood flow and microcirculation, nitric oxide-dependent neocapillary formation, and a partial increase in O_2 pressure in the local tissue, known as the Bohr Effect [20-22]. We have previously shown that transcutaneous application of CO_2 induced the release of O_2 from red blood cells, and increased O_2 pressure in treated tissues by causing an 'artificial Bohr effect' by the absorption of CO_2 into the human body [23]. We also demonstrated that transcutaneous CO_2 therapy increased peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) expression and the number of mitochondria in rat skeletal muscle [24]. In musculoskeletal tumors, we reported that this system could induce mitochondrial apoptosis by increasing the expression of PGC-1α in human MFH cells [25].

Therefore, we hypothesized that transcutaneous application of CO_2 may enhance the effect of chemotherapeutic agents by reducing the hypoxic environment of tumors, similar to that seen in skeletal muscles. Furthermore, the combination of transcutaneous CO_2 exposure and chemotherapy could increase the efficacy of treatment for human MFH. Here, we investigated the effect of oxygenation by transcutaneous application of CO_2 on the therapeutic efficacy of DOX to treat human MFH.

Materials and Methods

Cell culture

A human MFH cell line, Nara-H (ScienStuff Co., Nara, Japan) [26], was used in this study. Cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co., St Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich) and 100 U/ml penicillin/streptomycin solution (Sigma-Aldrich). Cells were maintained at 37°C in a humidified 5 % CO_2 atmosphere.

In vitro MFH cell experiments

To investigate the effect of altering the oxygen conditions of human MFH cells *in vitro*, Nara-H cells (ScienStuff Co.) [26] were incubated for 48 h in one of three different oxygen conditions: normoxic (20% O_2, 5% CO_2, 75% N_2), hypoxic (1% O_2, 5% CO_2, 94% N_2), or reoxygenated conditions. In the reoxygenated condition, cells were incubated under normoxic conditions for 24 h followed by 24 h of incubation under hypoxic conditions. After 48 h of incubation, total RNA and cell lysates were collected from cells, and the expression of VEGF mRNA and HIF-1α protein were evaluated by quantitative real-time PCR (qRT-PCR) and immunoblot analysis, respectively.

We also examined the effect of altering the oxygen conditions on the chemosensitivity of human MFH cells to DOX. After 24 h of incubation in one of three different oxygen conditions, medium was refreshed containing 1 μM of DOX (Adriamycin; 14-Hydroxydaunomycin, HCl, Merck, Darmstadt, Germany), and incubation was continued for a further 24 h. Then, the apoptotic activity of cells under each oxygen condition treated with DOX was assessed by DNA fragmentation assay.

Animal models

Male athymic BALB/c nude mice, aged 5 to 8 weeks, were obtained from CLEA Japan, Inc (Tokyo, Japan). Animals were maintained under pathogen-free conditions, in accordance with institutional principles. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine, and Kobe University Animal Experimentation Regulations (Permission number: P101203) and were approved by the Institutional Animal Care and Use Committee. Nara-H cells (ScienStuff Co.) [26] were injected into the dorsal, subcutaneous area of mice at a dose of 4.0×10^6 cells in 500 μl PBS as previously described [27].

Transcutaneous CO_2 treatment

Transcutaneous application of CO_2 was performed as previously described [26]. Briefly, the area of skin around the implanted tumor was covered with CO_2 hydrogel. This area was then sealed with a polyethylene bag, and 100% CO_2 gas was administered into the bag (Figure 1). Each treatment was performed for 10 min. Control animals were treated similarly, replacing CO_2 with room air.

In vivo MFH tumor studies

To investigate the effect of transcutaneous CO_2 treatment on the

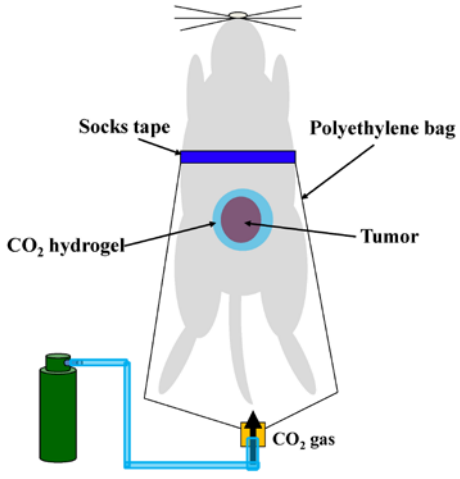

Figure 1: Transcutaneous application of CO_2 in an animal model of human MFH. The area of skin around the implanted tumor was covered with CO_2 hydrogel and sealed with a polyethylene bag, through which CO_2 gas was administered.

hypoxic conditions in MFH tumor tissues, we isolated tumors from mice at 0, 6 and 24 h after CO_2 treatment, and evaluated the expression of HIF-1α and VEGF by immunoblot analysis and qRT-PCR.

Next, we examined the effect of combination therapy of CO_2 and DOX treatment on human MFH *in vivo*. Twenty-four mice were randomly divided into four groups as follows: control group (n = 6); CO_2 group (n = 6); DOX group (n = 6); and combination (CO_2 + DOX) treatment group (n = 6). CO_2 (or room air as control) treatment commenced three days after MFH cell implantation, and was performed twice weekly for 2 weeks. In DOX and combination groups, mice were administered an intraperitoneal injection of 4 mg/kg DOX (Adriamycin; Merck), and CO_2 (or room air) treatment in the combination group was performed immediately after DOX injection. In the control group, the same procedure was performed but using PBS and room air, instead of DOX and CO_2 gas, respectively. The tumor volume and body weight in mice were monitored twice weekly until the end of the treatment. Tumor volume (V) was calculated as previously described [27], according to the formula $V = \pi/6 \times a^2 \times b$, where a and b represent the shorter and longer dimensions of the tumor, respectively. At the end of treatment, all tumors were excised from mice. For DNA fragmentation assays, the tumors were immediately treated to obtain a single cell suspension, and for other experiments, tumors were stored at -80°C until use. To assess the apoptotic activity of treated tumors, we performed immunofluorescence staining and a DNA fragmentation assay, and evaluated the expression of apoptosis related proteins, such as caspases and poly (ADP-ribose) polymerase (PARP), by immunoblot analyses.

Quantitative real-time PCR (qRT-PCR)

The mRNA expression of VEGF was analyzed by quantitative real-time PCR (qRT-PCR) [28]. Total RNA was extracted from MFH cells and implanted tumors by selective binding to a silica-gel-based membrane using an RNeasy Mini Kit, following the manufacturer's protocol (QIAGEN, Valencia, CA). First strand cDNA was reverse transcribed with 1 μg of total RNA and oligo dT primer by MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). qRT-PCR was performed in a 20 μl reaction mixture using SYBR Green Master Mix reagent (Applied Biosystems) on the ABI prism 7500 sequence detection system (Applied Biosystems). PCR conditions were as follows: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Pre-designed primers specific for human *VEGF* and human *β-actin* were obtained from Invitrogen (Carlsbad, CA). Primer sequences were as follows: human *VEGF*, 5' - CACATAGGAGAGATGAGCTTC - 3' (forward) and 5' -CCGCCTCGGCTTGTCACAT - 3' (reverse); human *β-actin*, 5' - GATCATTGCTCCTCCTGAGC - 3' (forward) and 5' - ACATCTGCTGGAAGGTGGAC - 3' (reverse). The relative expression of VEGF was calculated using the delta-delta Ct method, normalized to β-actin levels.

Immunoblot analysis

Cell lysates were prepared from MFH cells and implanted tumors using whole cell lysis buffer (Mammalian Protein Extraction Reagent; Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). Protein concentration was quantified using the Bradford Protein Assay reagent (Bio-Rad, Richmond, CA), and samples were processed using standard immunoblotting procedures [28]. Membranes were incubated overnight at 4°C with the following antibodies in Can Get Signal Solution 1 (Toyobo Co., Ltd, Osaka, Japan): anti-human HIF-1α anti-body (1:1000) (Cell Signaling Technology, Danvers, MA); anti-human cleaved caspase 3 antibody (1:1000) (Cell Signaling Technology); anti-human cleaved caspase 9 antibody (1:1000) (Cell Signaling Technology); anti-human cleaved PARP antibody (1:1000) (Cell Signaling Technology); and anti-human α-tubulin antibody (1:2000) (Sigma-Aldrich). Following washes, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase, and exposed with ECL Plus Western blotting detection system reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The protein levels were detected using the Chemilumino analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan).

DNA fragmentation assay

DNA fragmentation was evaluated using the Apo-Direct Kit according to the manufacturer's protocol (BD Pharmingen, Franklin Lakes, NJ). To obtain a single cell suspension from implanted tumors, tumors were minced and filtered through a cell strainer (BD Falcon, Bedford, MA). Erythrocytes were lysed in BD Pharm Lyse™ Lysing Buffer (BD Pharmingen), and the remaining cells were pelleted and resuspended in PBS. Single cell suspensions from MFH cells or implanted tumors were fixed with 1% (v/v) paraformaldehyde and resuspended in 70% (v/v) ice cold ethanol at a concentration of 1×10^6 cells/ml. Each cell pellet was resuspended in 50 μl of DNA Labeling Solution (Reaction Buffer: 10 μl, TdT Enzyme: 0.75 μl, FITC dUTP: 8.0 μl, distilled H_2O: 32.25 μl) and incubated for 60 min at 37°C. FITC-dUTP-labeled cells were analyzed by flow cytometry with a 520 nm Argon laser. Fluorescence intensity in each group was normalized to that in control group, and statistically analyzed.

Immunofluorescence staining

To assess the cellular apoptotic activity in treated tumors, we also performed immunofluorescence staining using Apo-Direct Kit following the manufacturer's protocol (BD Pharmingen). Images were obtained using a BZ-8000 confocal microscope (Keyence).

Immunohistochemical analysis

The excised tumors were embedded in OCT compound (Sakura Finetek Co, Tokyo, Japan), then frozen in a liquid nitrogen. Sections 10 μm thick were prepared on a cryostat and stored frozen at -70°C. Sections were air dried, fixed in 4% paraformaldehyde for 10 min at room temperature, and washed with PBS. After incubation with primary antibody overnight at room temperature, the sections were washed in PBS, and then treated with secondary antibody for an additional 30 min. For immunohistochemical staining, anti-human HIF-1α antibody (Santa Cruz BioTechnology Inc., Santa Cruz, CA) was used as the primary antibody followed by counterstain with hematoxylin and eosin (H&E).

Statistical analyses

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

Results

Reduced hypoxic conditions enhanced the chemotherapeutic effect of DOX on human MFH cells *in vitro*

We first examined the effect of altering the oxygen levels on human

MFH cells *in vitro* by culturing cells under three different oxygen conditions. After 48 h of incubation, protein expression of HIF-1α was strongly increased in cells under hypoxic conditions (1% O_2), whereas the expression was decreased in cells cultured under reoxygenated conditions (1% O_2 and 20% O_2) (Figure 2A). In addition, mRNA expression of VEGF, a downstream effector of HIF-1α, was significantly decreased in reoxygenated MFH cells compared with hypoxic MFH cells, similar to that for HIF-1α expression ($p < 0.05$, Figure 2B). Next, we evaluated the effect of alternative oxygen conditions on the chemotherapeutic efficacy of DOX by measuring the apoptotic activity in cells from the three different groups. DOX treatment induced an apoptotic effect on MFH cells under normoxic conditions, but the apoptotic activity was strongly decreased in hypoxic MFH cells (Figure 2C). Moreover, the apoptotic activity in reoxygenated cells was higher than that in hypoxic cells (Figure 2C). These results suggest that hypoxic conditions decrease the chemotherapeutic effect of DOX on human MFH cells *in vitro*, and that by reducing hypoxic conditions we can enhance the beneficial effects of DOX.

Transcutaneous application of CO_2 decreased the expression of HIF-1α and VEGF in MFH tumors

To determine the effect of transcutaneous application of CO_2 on the oxygen conditions in human MFH cells *in vivo*, we developed a murine model of human MFH by transplanting the Nara-H cell line into the dorsal subcutaneous area of mice. After 0, 6 and 24 h of transcutaneous CO_2 treatment, we evaluated the expression of HIF-1α and VEGF mRNA in treated tumors by immunoblot analysis and qRT-PCR, respectively. Transcutaneous application of CO_2 decreased the expression of HIF-1α protein (Figure 3A) and VEGF mRNA ($p < 0.05$, Figure 3B) in MFH tumors in a time-dependent manner. This indicates that transcutaneous CO_2 exposure reduced the hypoxic conditions in human MFH *in vivo*.

Transcutaneous CO_2 treatment enhanced *in vivo* antitumoral effects of DOX on human MFH cells

Finally, we evaluated the *in vivo* effects of combination therapy using transcutaneous CO_2 and DOX treatment on human MFH cells. Three days after MFH cell implantation, mice were randomly assigned to one of four groups as follows: CO_2 group; DOX group; combination (CO_2 + DOX) treatment group; and control group, and each treatment were performed twice weekly for 2 weeks. At the end of the study, the tumor volume in the CO_2 (2013 ± 166 mm^3) and DOX groups (1927 ± 91 mm^3) was significantly smaller compared with the control group (3960 ± 166 mm^3) ($p < 0.01$, Figure 4A). In the combination group, we observed a significant decrease in tumor volume compared with the control group at day 7 ($p < 0.05$). In addition, after 2 weeks of treatment the tumor volume from the combination group was significantly reduced (1106 ± 105 mm^3) compared to the control, CO_2 and DOX groups ($p < 0.01$, Figure 4A). A significant difference in body weight was observed in the DOX and combination groups compared with control groups ($p < 0.05$, Figure 4B), however transcutaneous CO_2 therapy alone did cause any observable negative side effects in terms of body weight loss in mice (Figure 4B). In immunohistochemical analysis, HIF-1α expression was observed in control and DOX groups, but not in CO_2 and combination groups (Figure 4C). The result indicates that our CO_2 treatment reduces hypoxic condition that should enhance the chemosensitivity, in treated tumors. We also evaluated the apoptotic activity in treated tumors by immunofluorescence staining, DNA fragmentation assay and immunoblot analyses. Immunofluorescence staining showed that in CO_2 and DOX treatment groups, increased numbers of apoptotic cells were detected compared with the control group. However, there were markedly increased numbers of apoptotic cells in the combination group compared with the other three groups (Figure 5A and 5B). Consistent with the immunofluorescence results, apoptotic activity was significantly increased in the CO_2 and DOX groups compared with that in control group ($p < 0.05$, Figure 5C and 5D). And, the increase in apoptotic activity was more significant in the combination group compared with that in the control, CO_2 and

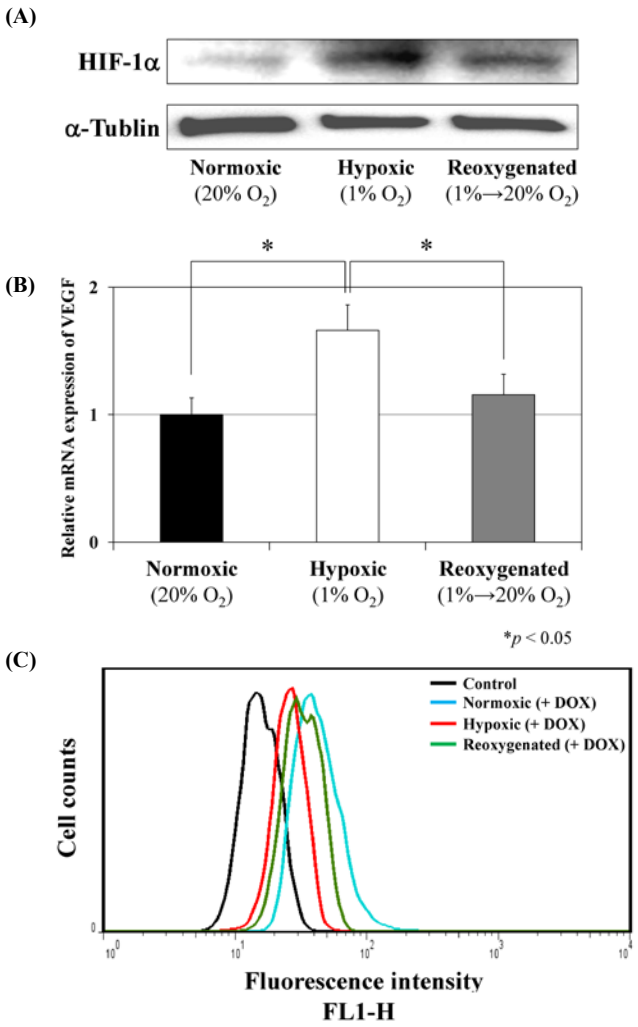

Figure 2: Effect of altering oxygen conditions on human MFH cells *in vitro*. A human MFH cell line, Nara-H was incubated for 48 h under one of three different oxygen conditions: normoxic (20% O_2, 5% CO_2, 75% N_2); hypoxic (1% O_2, 5% CO_2, 94% N_2); or reoxygenated conditions. In the reoxygenated condition, cells were incubated under normoxic conditions for 24 h followed by 24 h exposure of hypoxic conditions. (A) Immunoblot analyses showed that HIF-1α expression was increased in hypoxic conditions compared with normoxic conditions, but was decreased in reoxygenated conditions. (B) mRNA expression of VEGF in cells cultured in one of three different oxygen conditions was evaluated by qRT-PCR. VEGF mRNA expression was significantly increased in hypoxic MFH cells compared with normoxic and reoxygenated cells (*$p < 0.05$ versus both normoxic and reoxygenated conditions). (C) Apoptotic effect of DOX treatment on human MFH cells under three different oxygen conditions was assessed DNA fragmentation assay by FACS analyses. Under normoxic conditions, DOX treatment induced apoptosis of human MFH cells (*light blue*). Apoptotic activity was strongly decreased in hypoxic cells (*red*), and the activity was recovered under reoxygenated conditions (*green*).

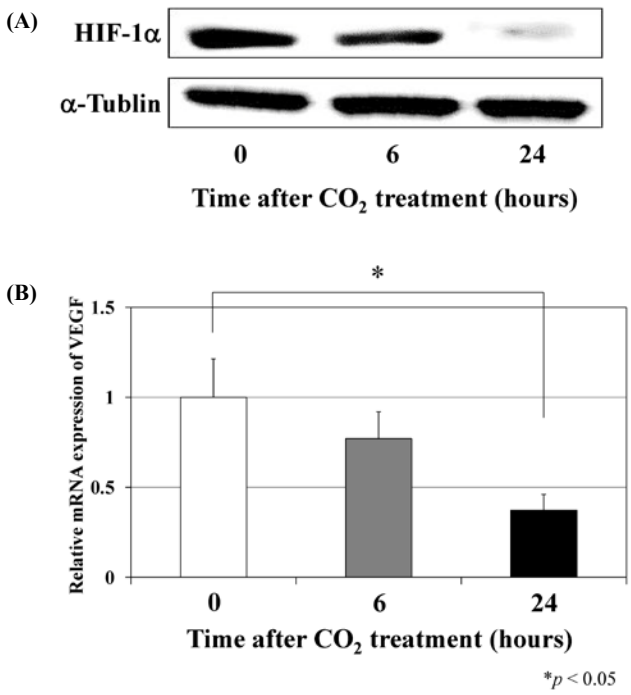

Figure 3: Effect of transcutaneous CO_2 application on oxygenation in a mouse model of human MFH. Mice were treated with CO_2 or control air three days after MFH cell implantation. Tumors were isolated from mice at 0, 6 and 24 h after CO_2 gas treatment. (A) Protein expression of HIF-1α decreased time-dependently by 24 h post-transcutaneous CO_2 treatment. (B) Consistent with HIF-1α expression, mRNA expression of VEGF in tumors showed a time-dependent decrease at 24 h post-CO_2 treatment. The expression after 24 h of treatment was significantly decreased compared with before treatment (*$p < 0.05$).

DOX groups ($p < 0.01$, Figure 5C and 5D). By immunoblot analyses, we observed increased expression of caspase 3, 9 and PARP cleavage products in all three treatment groups, but not in the control group. The highest protein expression levels were detected in the combination group (Figure 5E).

Discussion

MFH is the most common high-grade soft tissue sarcoma to occur in late adult life. Advances in the treatment of MFH have led to multidisciplinary treatments, including surgery, chemotherapy, and radiation therapy, resulting in great improvements in the quality of life for patients with MFH. However, MFH is still largely resistant to conventional chemotherapy [29]. DOX, an extensively used anticancer agent, is a high-affinity, sequence selective DNA intercalating agent that targets topoisomerase II. It functions by inducing DNA damage and the formation of reactive oxygen species (ROS) are primarily responsible for its cytotoxic effects [30]. DOX has been employed in many chemotherapeutic regimens for the treatment of acute leukemia, non-Hodgkin's lymphoma and different types of solid tumors [30,31]. However, the antitumoral effect of DOX on MFH has been extremely poor [31]. Unfortunately, DOX can cause a number of unwanted side effects, especially cardiac toxicity [32]. In the current study, we observed severe weight loss in mice treated with 6 or 8 mg/kg DOX (data not shown), and therefore treated mice *in vivo* with 4 mg/kg DOX, although 4 mg/kg DOX has a reduced antitumor effect on human MFH.

The hypoxic microenvironment inside solid tumors is a major cause of chemoresistance [33], and it has been suggested that chronically hypoxic cells are more resistant to various chemotherapeutic agents

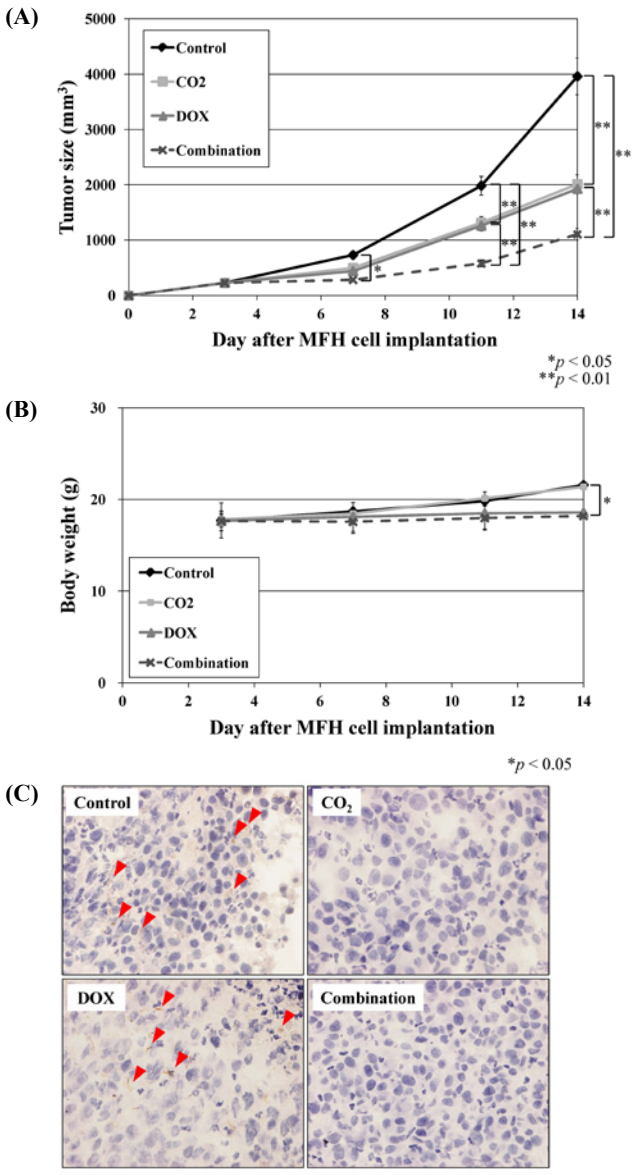

Figure 4: *In vivo* effects of combination therapy using transcutaneous CO_2 treatment and DOX on human MFH. Three days after MFH cell implantation, mice were randomly assigned to four groups: control group, CO_2 group, DOX group, and combination (CO_2 + DOX) treatment group. Each treatment was performed twice weekly for 2 weeks, and the tumor volume (A) and body weight (B) in mice were monitored until the end of the treatment. (A) At day 7, we observed a significant decrease in tumor volume in the combination group compared with the control group (*$p < 0.05$). At 14 d, the tumor volume in the CO_2 or DOX groups was significantly decreased compared with the control group (**$p < 0.01$ for CO_2 or DOX groups versus control group). The tumor volume in the combination group was significantly reduced compared with the other three groups (**$p < 0.01$). (B) We observed significant body weight loss in both DOX and combination treated groups compared to groups without DOX treatment (*$p < 0.05$, Figure 4B). Mice treated with transcutaneous CO_2 did not develop any observable negative side effects. (C) Immunohistochemical staining of HIF-1α in treated tumors. The expression of HIF-1α (*Red arrows*) was observed in tumors in control and DOX groups, but not in CO_2 and combination groups.

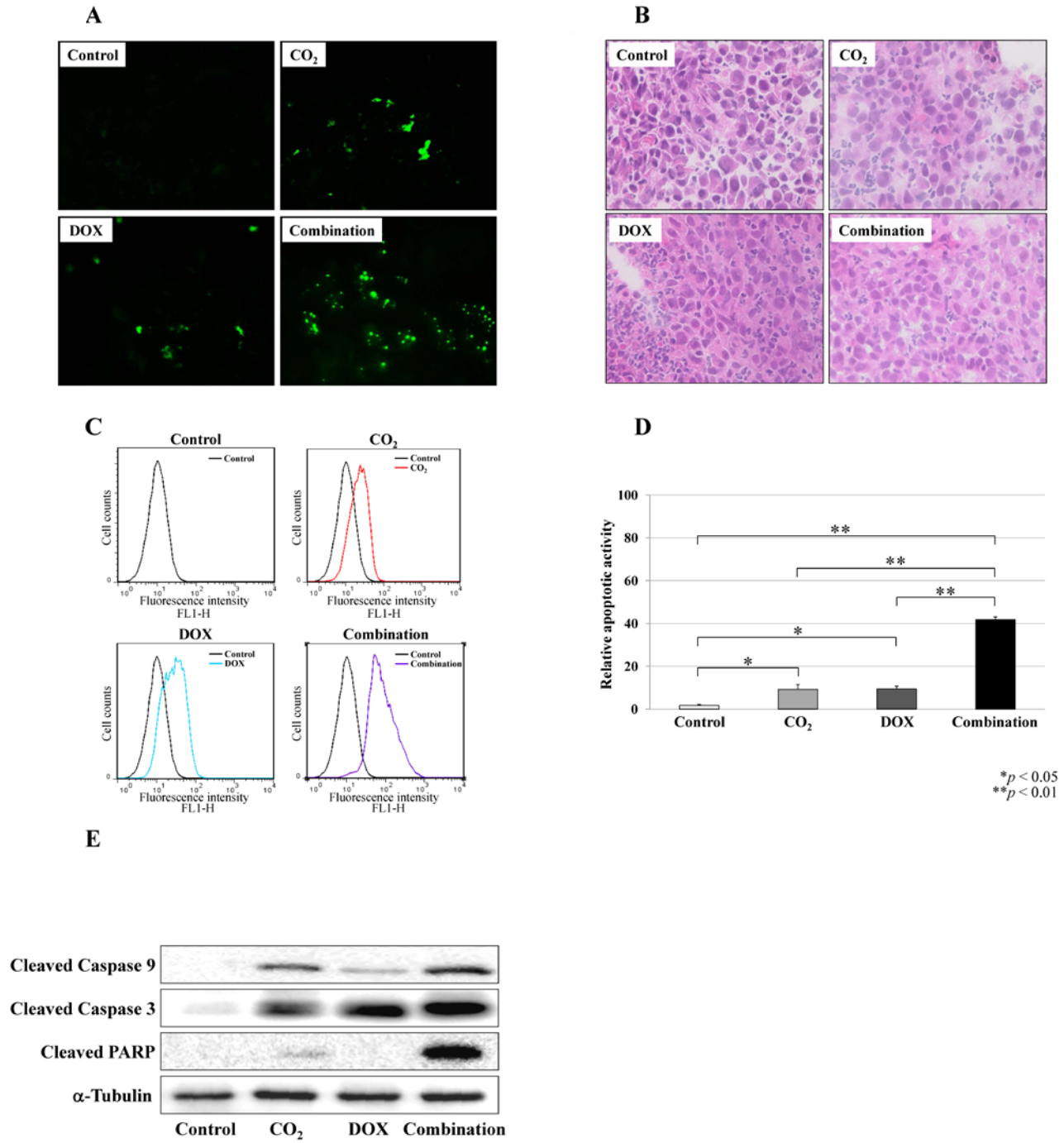

Figure 5: Effect of the combination therapy using CO_2 treatment and DOX on human MFH cell apoptosis. We examined the apoptotic activity in treated tumors by immunofluorescence staining, DNA fragmentation assay and immunoblot analysis. (A) Immunofluorescence staining showed that more apoptotic cells were detected in the CO_2 or DOX groups compared with the control group, whereas the number of apoptotic cells was dramatically increased in the combination group. (B) H&E staining matching the immunofluorescence staining. (C) DNA fragmentation was assessed by flow cytometry in the control (*black*), CO_2 (*red*), DOX (*light blue*), and combination groups (*purple*) at the end of treatment (14 d). (D) Fluorescence intensity in each group was normalized to that in control group, and statistically analyzed. Consistent with the immunofluorescence results, apoptotic activity in the CO_2 and DOX groups was significantly increased compared with that in control group (*$p < 0.05$). The apoptotic activity in the combination group was significantly increased compared with the control, CO_2 and DOX groups (**$p < 0.01$). (E) Immunoblot analyses determined that increased expression of the cleavage products of caspase 3, 9 and PARP occurred in the CO_2, DOX, and especially the combination group compared with the control group.

than normally proliferating cells [9]. Smith et al. [7], reported that chronically hypoxic cells are resistant to the cytotoxic action of DOX, and that resistance is decreased in reoxygenated cells. In the hypoxic response pathway, HIF-1α plays a central role by regulating a wide range of hypoxia-related molecules [11]. HIF-1α has been reported to regulate the transcription of ATP-binding cassette (ABC) transporter genes, and to contribute to the multidrug resistance of cancer cells [34]. Overexpression of the ABC transporter family of genes, including P-glycoprotein and multidrug resistance related proteins, has been demonstrated to cause efflux of intracellular anticancer drugs to the extracellular space, resulting in promotion of chemoresistance [34,35]. HIF-1α also regulates VEGF expression in hypoxic cells [36]. VEGF production by hypoxic malignant cells is associated with angiogenesis, increases in paracrine/autocrine growth factor release, enhancement of cell motility, promotion of metastasis, inhibition of apoptosis, and chemoresistance in various cancer cells, including soft tissue sarcomas [37]. In the current study, we observed increased expression of HIF-1α and VEGF in hypoxic MFH cells *in vitro*. In addition, we observed that MFH cells grown under hypoxic conditions were more resistant to DOX compared to those in normoxic conditions, and that DOX-resistance was decreased in reoxygenated cells. The results are consistent with a previous report [7], and suggest that hypoxia contributes to chemoresistance in human MFH cells.

Several treatment modifications to either overcome hypoxia or to specifically target hypoxic cells have been investigated [19,38,39]. These modalities were often combined with chemotherapy [38,39], and an increase in local and regional tumor control was achieved by chemotherapy applied under hyperbaric oxygen conditions [39]. Unfortunately, attempts to improve tumor oxygen during therapy have not yielded clinically compelling results. CO_2 therapy refers to the transcutaneous administration of CO_2 for therapeutic purposes [23,24], and its therapeutic effect has been explained by an increase in the pressure of O_2 in treated tissues, known as the Bohr effect [23]. We have previously shown that use of a transcutaneous application of CO_2 system causes the absorption of CO_2 and increases O_2 pressure in the treated tissue, potentially causing an 'artificial Bohr effect' [23]. Therefore, we employed the transcutaneous CO_2 system to reduce tumor hypoxia, and investigated the effect of CO_2 therapy combined with DOX treatment on human MFH *in vivo*. In the current study, we observed that use of a transcutaneous CO_2 system significantly decreased the expression of both HIF-1α and VEGF in human MFH tumor tissues, suggesting that the system reduced the hypoxic conditions in human MFH. Furthermore, transcutaneous CO_2 therapy alone had an antitumoral effect, and increased the chemotherapeutic effect of DOX on MFH tumor growth *in vivo*, with no observable effects on body weight. The results strongly indicate that the transcutaneous CO_2 system presented here has antitumor effects and can enhance the chemosensitivity of tumor cells by reducing the local hypoxic conditions.

In summary, this is the first report to our knowledge that demonstrates transcutaneous application of CO_2 reduces tumor hypoxia, and increases the chemotherapeutic effect of DOX on *in vivo* models of human MFH. Although further studies are needed to elucidate the mechanisms of the effects of the transcutaneous CO_2 system on tumors, our data indicate that this system may represent a therapeutic breakthrough for various human malignancies with chemoresistance.

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Conflict of Interest Statement

All authors have no conflict of interest.

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