Optimization of antitumor treatment conditions for transcutaneous CO_2 application: An *in vivo* study

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Abstract. Carbon dioxide (CO_2) therapy can be applied to treat a variety of disorders. We previously found that transcutaneous application of CO_2 with a hydrogel decreased the tumor volume of several types of tumors and induced apoptosis via the mitochondrial pathway. However, only one condition of treatment intensity has been tested. For widespread application in clinical antitumor therapy, the conditions must be optimized. In the present study, we investigated the relationship between the duration, frequency, and treatment interval of transcutaneous CO_2 application and antitumor effects in murine xenograft models. Murine xenograft models of three types of human tumors (breast cancer, osteosarcoma, and malignant fibrous histiocytoma/undifferentiated pleomorphic sarcoma) were used to assess the antitumor effects of transcutaneous CO_2 application of varying durations, frequencies, and treatment intervals. In all human tumor xenografts, apoptosis was significantly induced by CO_2 treatment for ≥ 10 min, and a significant decrease in tumor volume was observed with CO_2 treatments of >5 min. The effect on tumor volume was not dependent on the frequency of CO_2 application, i.e., twice or five times per week. However, treatment using 3- and 4-day intervals was more effective at decreasing tumor volume than treatment using 2- and 5-day intervals. The optimal conditions of transcutaneous CO_2 application to obtain the best antitumor effect in various tumors were as follows: greater than 10 min per application, twice per week, with 3- and 4-day intervals, and application to the site of the tumor. The results suggest that this novel transcutaneous CO_2 application might be useful to treat primary tumors, while mitigating some side effects, and therefore could be safe for clinical trials.

Introduction

Malignant tumors are the primary cause of death from disease in developed nations (1). The main treatment options for tumors are surgery and chemo- and radiation therapy (2), which yield good results (3). Nonetheless, these treatments also have various side effects (4). Therefore, there is a need for novel antitumor therapies that are not associated with side effects or complications.

Carbon dioxide (CO_2) has been mainly used as a treatment for peripheral vascular disorders (5). The benefits of a carbonated spa have long been known in Europe (6), and are still enjoyed in many countries (7). Bathing in artificial CO_2-enriched water has been shown to improve ischemic limb symptoms (8). CO_2 exerts therapeutic effects by stimulating blood flow and microcirculation (9) to increase partial O_2 pressure in local tissue, which is known as the Bohr effect (10). We previously investigated whether the Bohr effect can be induced by transcutaneous CO_2 application using 100% CO_2 gas and CO_2 absorption-enhancing hydrogel in humans (11). We showed that transcutaneous application of CO_2 to the lower limbs in rats for three months activated the expression of peroxisome proliferator-activated receptor gamma co-activator 1α in the tibialis anterior muscle, and increased the number of mitochondria in skeletal muscles, even in malignant tumor tissues (12-14). We also found that this CO_2 treatment could induce mitochondrial apoptosis in human malignant fibrous histiocytoma/undifferentiated pleomorphic sarcoma (MFH/UPS) (13), murine osteosarcoma (15), and human oral squamous cell carcinoma (14) without any side effects such as loss of body weight and induction of metastasis.

These results suggest that percutaneous CO_2 treatment can be used as an antitumor therapy. However, before initiating clinical trials, the optimal conditions, including the duration and frequency of transcutaneous CO_2 application, must be established to decrease tumor volume and induce apoptosis in tumor cells. The present study represents a preclinical test to

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investigate the antitumor effects of transcutaneously applied CO$_2$, against three types of human tumors, with regard to treatment conditions including duration, frequency, and site of CO$_2$ exposure, in mouse xenograft models.

Materials and methods

Cell lines. MDA-MB-231 human breast cancer cells (American Type Culture Collection (ATCC), Rockville, MD, USA) (16), MG63 human osteosarcoma cells (ATCC) (17), and Nara-H human MFH/UPS cells (ScienStuff Co., Nara, Japan) (13,18) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin/streptomycin solution (all from Sigma-Aldrich, St. Louis, MO, USA) at 37˚C in a humidified atmosphere of 5% CO$_2$.

Animal models. Male, athymic BALB/c nude mice (5-8 weeks old) were obtained from CLEA Japan (Tokyo, Japan). The animals were maintained under pathogen-free conditions; experiments were performed in accordance with the Guidelines for Animal Experimentation of Kobe University Graduate School of Medicine and Kobe University Animal Experimentation Regulations (permission nos. P110905-R1 and P-101203) and were approved by the Institutional Animal Care and Use Committee. To create human tumor xenograft models, MDA-MB-231 ($3.0x10^6$), MG63 ($5.0x10^6$), and Nara-H ($5.0x10^6$) cells in 500 μl of phosphate-buffered saline (PBS) were injected into the dorsal subcutaneous area of mice, as previously described (13-15). CO$_2$ treatment was initiated after cell implantation when the tumors were of a measurable size. Tumor volume and body weight were monitored twice weekly until the end of the treatment. Tumor volume was calculated as described previously (13,18), using the formula: $V = \pi/6 \times a^2 \times b$, where a and b represent the shorter and longer dimensions of the tumor, respectively.

Transcutaneous CO$_2$ application. For treatment, CO$_2$ was administered transcutaneously, as described previously (12-15). Briefly, the area of skin around the implanted tumor was treated with a CO$_2$ hydrogel. The area was then sealed with a polyethylene bag, and 100% CO$_2$ gas was delivered into the bag.

Effect of treatment duration on different tumor types. Based on the average tumor volume after the tumors reached a measurable size, 24 mice for each cell line were randomly divided into four groups of six mice each; these groups received the treatment for 0, 5, 10, or 20 min per application, with the applications performed twice weekly for 2 weeks (12-15). Results are shown as the ratio of the final tumor volume to the corresponding pretreatment value.

Effect of treatment frequency and interval. In total, 18 mice with Nara-H cell implantation were randomly divided into three groups of six mice each; one group was a control, whereas the other groups received the treatment twice per week or five times per week. Mice were treated with CO$_2$ for 10 min at the specified frequency for 1 week starting 4 days after cell implantation (Fig. 1A). At the completion of treatment, the ratio
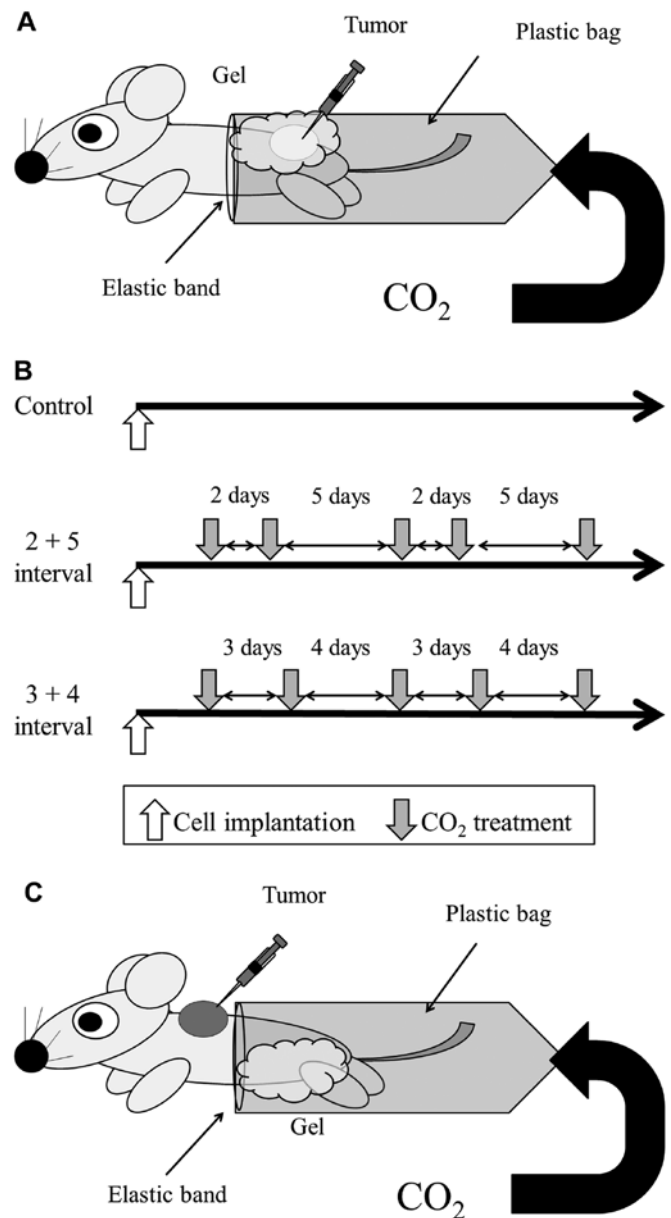

Figure 1. Experimental conditions of CO$_2$ application. (A) General tumor implantation site and CO$_2$ application. (B) Time schedule of CO$_2$ treatment intervals. (C) Tumor implantation into the dorsal subcutaneous area and CO$_2$ application to the ventral area.

of tumor volume after the treatment compared to that before the treatment was calculated.

To assess the effect of treatment interval, CO$_2$-treatment was performed twice per week for 2 weeks, using two different treatment intervals: 2 and 5 day intervals (2 + 5 interval group) or 3 and 4 day intervals (3 + 4 interval group), as shown in Fig. 1B. For each treatment interval, 18 mice with Nara-H cell implantation were divided into two groups of nine mice each as follows: control group for the 2 + 5 interval (n=9), CO$_2$-treatment group for the 2 + 5 interval (n=9), control group for the 3 + 4 interval (n=9), and CO$_2$-treatment group for the 3 + 4 interval (n=9). At the completion of treatment, the ratio of tumor volume relative to that of the control was calculated.

Effect of treatment application site. We also investigated whether CO$_2$ application had antitumor effects at distant sites.
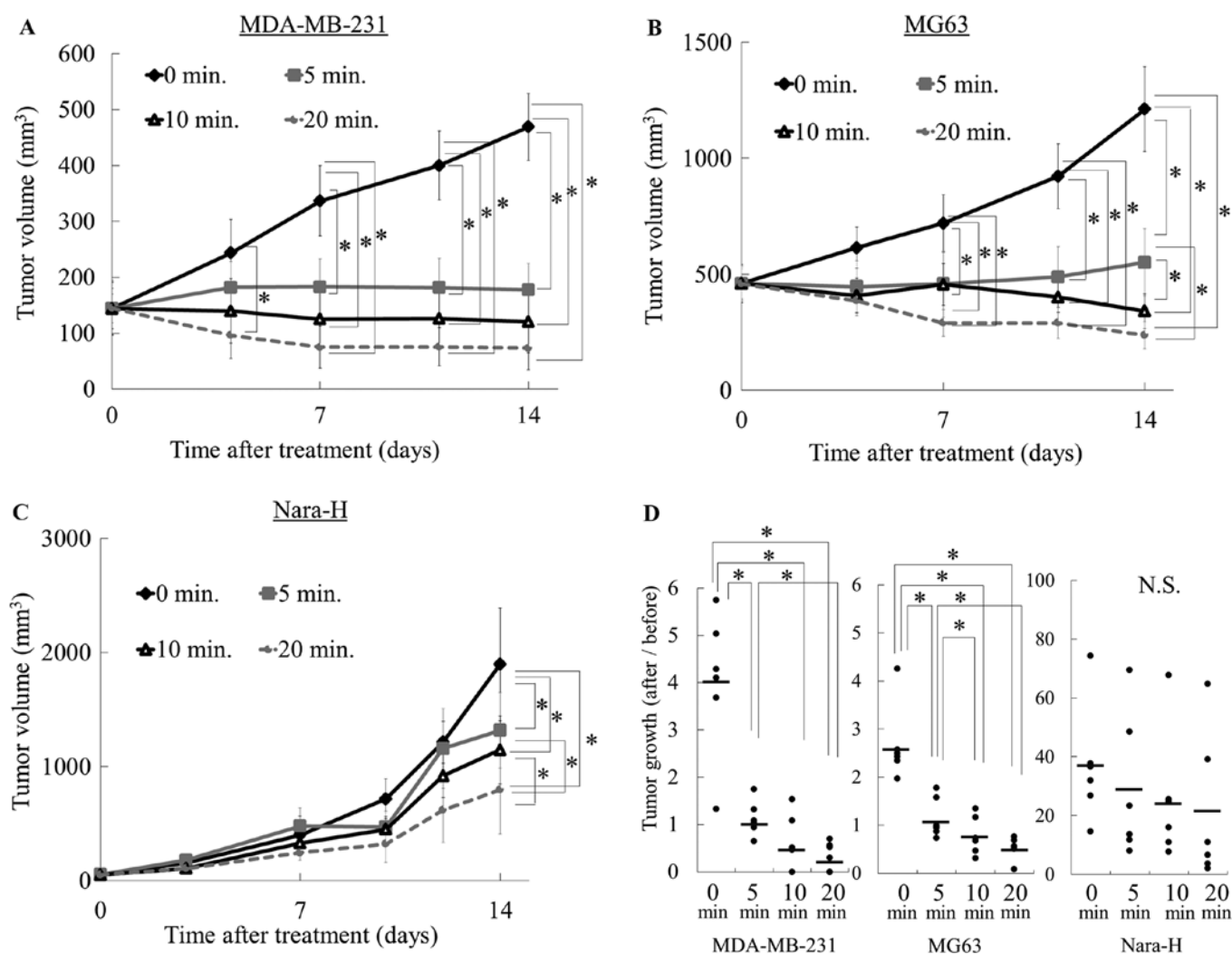
Figure 2. Tumor volume after CO_2 application with various treatment durations in mouse xenograft models. (A) Breast cancer, (B) osteosarcoma, and (C) MFH/UPS tumor growth with transcutaneous CO_2 administered for different durations. (D) Tumor volume was monitored until the end of the experiment and was compared to corresponding pre-treatment values. Data represent the mean ± standard error (SE). *P<0.05.

Nara-H cells were implanted into the upper back of 12 mice, and the mice were randomly divided into two groups of six mice each: the control and CO_2 groups. After 4 days of cell implantation, treatment with CO_2 or air (as control) was applied to the abdominal region, which was a location completely different from that of the implanted tumor (Fig. 1C). CO_2-treatment was performed transcutaneously for 10 min each, twice weekly for 2 weeks.

DNA fragmentation assay. DNA fragmentation was evaluated using the APO-Direct kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Briefly, upon completion of the treatments, the implanted tumors were excised, minced, and filtered through a cell strainer (BD Biosciences) to obtain single-cell suspensions. Erythrocytes were lysed in lysis buffer (BD Biosciences), and the remaining cells were pelleted and re-suspended in PBS. Single-cell suspensions were fixed with 1% (v/v) paraformaldehyde and re-suspended in 70% (v/v) ice-cold ethanol at a concentration of 1x10^6 cells/ml. Each cell pellet was re-suspended in 51 μl of DNA labeling solution, and was incubated for 60 min at 37°C. FITC dUTP-labeled

cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences) with a 488 nm argon laser (12,13,18).

Statistical analysis. Analysis of variance with a post-hoc test was performed to compare continuous values. Differences were considered significant at P<0.05. Data are presented as the mean ± standard error (SE). For normally distributed data, the two-tailed t-test was used for comparisons between groups.

Results

CO_2 administration for at least 10 min reduces tumor volume. We investigated the optimal CO_2 administration time for inhibiting breast cancer, osteosarcoma, and MFH/UPS growth *in vivo*, using murine xenograft models. CO_2-treatment times of 5, 10, or 20 min reduced tumor volume relative to the control group (0 min) in MDA-MB-231 breast cancer and MG63 osteosarcoma mice (P<0.05); however, there were no differences among the three treatment groups using the MDA-MB-231 model (Fig. 2A) or between the 10- and 20-min groups using the MG63 model (Fig. 2B). In the Nara-H MFH/UPS model, a significant difference compared to the control was observed

Table I. Antitumor effect after CO_2 application with various treatment durations in each mouse.

Cell line	Response	0 min	5 min	10 min	20 min
MDA-MB-231	Reduced[a]	0/6	2/6	4/6	6/6
(Breast cancer)	Lost	0/6	0/6	1/6	2/6
MG63	Reduced[a]	0/6	2/6	4/6	6/6
(Osteosarcoma)	Lost	0/6	0/6	0/6	0/6
Nara-H	Reduced[a]	0/6	0/6	0/6	0/6
(MFH/ UPS)	Lost	0/6	0/6	0/6	0/6

Effectiveness of transcutaneous CO_2 application in reducing the growth of MDA-MB-231, MG63, and Nara-H cell-derived tumors. [a]'Reduce' showed before final tumor volume for pre-treatment and 'Lost' showed tumor mass disappeared.

Figure 3. Apoptotic activity after CO_2 applications of various durations. Detection of apoptosis in MFH/UPS cells using the DNA fragmentation assay following transcutaneous CO_2 application for 0, 5, 10, and 20 min. (A) Representative scatter plots of apoptotic cells detected by flow cytometry. (B) Quantitative analysis of data shown in (A). Data represent the mean ± standard error (SE). *P<0.05, **P<0.01.

in the 5-, 10-, and 20-min treatment groups, but only at the end of treatment (P<0.05), and there was no significant difference among the groups during the treatments (Fig. 2C). Body weight did not significantly change during the treatments in all mice implanted with any of the cell lines, and there was no evidence of pulmonary metastasis or symptoms of kidney damage such as hematuria (data not shown). These results indicate that CO_2 administration has a time-dependent inhibitory effect on breast cancer, osteosarcoma, and MFH/UPS growth *in vivo* without obvious side effects.

In the current study, the most significant antitumor effect was observed in the MDA-MB-231 breast cancer model. At the end of treatment, all treated tumors in the MDA-MB-231 model were smaller than pre-treated tumors, and notably, two of the six tumors in the 20-min group had disappeared. Similarly, in the osteosarcoma model, tumor volumes in both the 10- and 20-min treatment groups decreased compared to the pre-treatment volume. In contrast, when using the MFH/UPS cell model, the tumor volume was not reduced compared to the pre-treatment volume, although tumor growth was suppressed at all three treatment times relative to that of the control (Fig. 2D and Table I). We assessed the effect of transcutaneous CO_2 application on apoptotic activity in the MFH/UPS model and found that CO_2-treatment, with all three treatment durations (5, 10, and 20 min), strongly increased the rate of apoptosis, with a greater increase observed with longer treatment durations (Fig. 3A and B).

CO_2 administration twice per week at intervals of fewer than 4 days is optimal for inhibiting tumor growth. To determine the optimal frequency of transcutaneous CO_2 application, mice were treated with CO_2 at a frequency of twice or five times per week. The tumor volumes after treatment twice or five times per week were smaller compared to those of the control mice, and the antitumor effect was not affected by treatment frequency; i.e., the effect was the same regardless of whether the frequency was twice or five times per week (Fig. 4A). These results indicate that transcutaneous CO_2 application at twice per week has a significant antitumor effect.

Using a treatment frequency of twice per week, we also evaluated the differences in the antitumor effect according
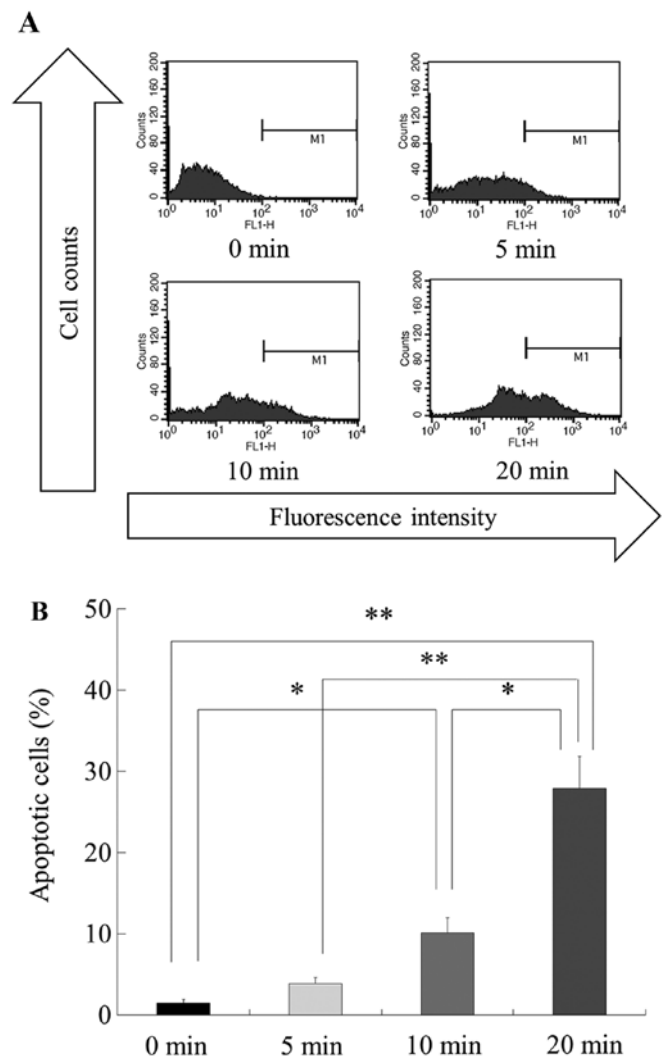
to the treatment interval, i.e., an interval of 2 and 5 days (2 + 5 interval) or 3 and 4 days (3 + 4 interval). As shown in Fig. 4B, the tumor volume in the 3 + 4 interval group was significantly lower than that in both the control and 2 + 5-interval groups, whereas the volume in the 2 + 5 interval group was not significantly different from that of the control group. These results indicate that the treatment interval of transcutaneous CO_2 application should be fewer than 4 days with a treatment frequency of twice per week.

Transcutaneous CO_2 exerts an antitumor effect at the site of application. To determine whether transcutaneous CO_2 application has local or systemic antitumor effects, tumor cells were injected into the upper back of mice (Fig. 1C), and CO_2 was applied to the abdominal region of the body for 10 min, twice per week for 2 weeks. As shown in Fig. 5, there was no reduction in tumor volume with this treatment when compared to that in control mice, indicating that local application of CO_2 at the tumor site can induce antitumor effects,

Figure 5. MFH/UPS tumor volume after transcutaneous CO_2 application at a distant site. Tumor volume was monitored until the end of the experiment. Data represent the mean ± standard error (SE). N.S., not significant.
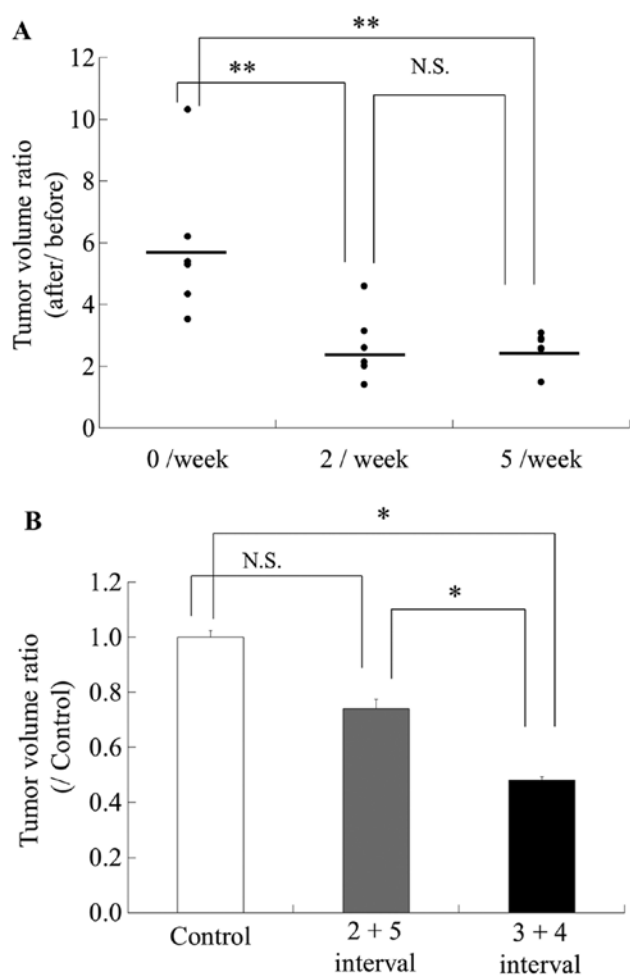
Figure 4 Antitumor effect after CO_2 applications of various treatment times and intervals. (A) Growth in tumor volume as a function of the duration of transcutaneous CO_2 application in an MFH/UPS xenograft model. CO_2 treatment was administered twice or five times per week. (B) Tumor growth ratios for the indicated treatment intervals. Data represent mean ± standard error (SE). *P<0.05, **P<0.01; N.S., not significant.

but application at a site distant from the tumor cannot induce antitumor effects.

Discussion

To obtain the best effect, new treatments for diseases including cancer must meet certain criteria; i.e. administration of the effective drug should be performed at the appropriate dose and time via the ideal delivery route in each patient (19). In this study, we investigated the appropriate use of transcutaneous CO_2 application as an antitumor therapy by evaluating its effects on different tumor types, testing various administration frequencies and intervals, and assessing whether the treatment effects were local or systemic. We found that CO_2-treatment was most effective against local tumors when applied transcutaneously for at least 10 min each, twice per week, with an interval of 3 and 4 days, and these conditions yielded no discernable side effects.

CO_2-treatment had little effect when applied for a short duration (5 min) in each treatment, whereas a longer treatment time (20 min) markedly increased the rate of apoptosis in human tumors. Additionally, in a preliminary study, no further
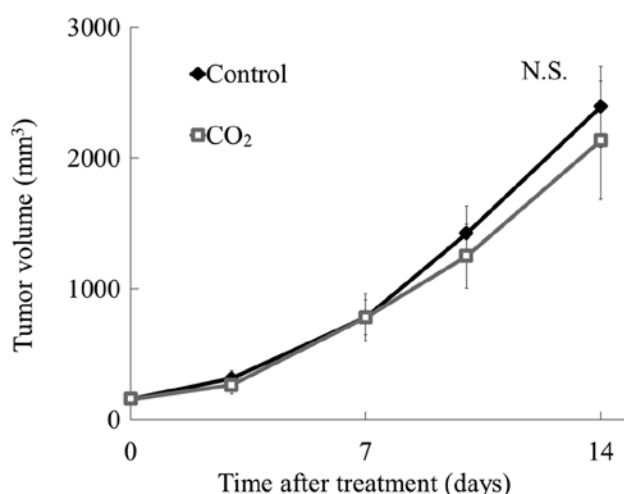
decrease in tumor volume was observed with the 1-h treatment compared to the 20-min treatment in the Nara-H xenograft model (data not shown). A DNA fragmentation assay also showed that the induction of apoptosis by CO_2 application was time-dependent, with a maximal increase observed for the 20-min treatment. These observations strongly suggest that the optimal treatment time for transcutaneous CO_2 application is 20 min for each treatment, and that a treatment time of at least 10 min should be used. For the CO_2 therapy, there was no difference in effectiveness of frequencies of twice and five times per week; in addition, a twice-weekly treatment regimen was sufficient to inhibit *in vivo* tumor growth, even in MFH/UPS, which is a high-grade sarcoma cell line. Additionally, using a treatment frequency of twice per week, tumor volumes were smaller with a treatment interval of 3 and 4 days than with a treatment interval of 2 and 5 days. We also found that transcutaneous CO_2 application was ineffective when applied at a distant site relative to the tumor location. Therefore, the CO_2 therapy should be applied to the body surface close to the tumor location. We previously developed an alternative system for CO_2 application that can access deep-seated tumors using intra-arterial infusion of saturated CO_2 solution (20). These findings suggest that continuous treatment, twice per week with an interval of 3 + 4 days, applied to the local site of the tumor, provides the best antitumor effect for transcutaneous CO_2 application.

Chemo- and radiation therapies are effective for many types of malignant tumors (4,21). However, these therapies at high dosages can damage not only malignant tumor cells, but also healthy cells and tissues (21-22), often resulting in side effects and complications (21). Specifically, chemotherapeutic drugs such as doxorubicin, ifosfamide, and cisplatin are known to cause myelosuppression, cardio- and nephritic toxicity, cystitis hemorrhage, and nausea (4,22), whereas radiation therapy is associated with radiodermatitis, myelosuppression, and radiation disease (21,23-25). In this study, transcutaneous CO_2 application did not result in any side effects such as body weight loss, cystitis hemorrhage, dermatitis, cardiac arrest, or metastasis even with long and/or frequent treatment. This

indicates that CO$_2$ therapy is likely safe for treating patients with malignant tumors. In addition, we previously showed that CO$_2$ had synergistic effects by reducing hypoxia when used in combination with chemo- (26) and radiation therapies (27); however, it remains unclear exactly how CO$_2$-treatment should be used in combination with these types of therapy.

This study had several limitations. First, in the current study, the antitumor effects of transcutaneous CO$_2$ application were tested in only three cancer types, with one cell line for each cancer type. Our previous studies (13-15,26-27) suggest that transcutaneous CO$_2$ application should be effective in various types of cancers; however, we need to verify the optimal conditions using more cancer types and several different cell lines for each type. Second, we confirmed the antitumor effects of CO$_2$ therapy on human cancer cell types; however, the experiments were performed using animal models. The antitumor effects of transcutaneous CO$_2$ application in humans should now be evaluated in clinical studies.

In conclusion, our findings indicate that local transcutaneous CO$_2$ application for at least 10 min, twice per week, with an inter-treatment interval of fewer than 4 days, and with administration to the tumor site can be an effective therapeutic approach for various types of cancers and sarcomas. The results strongly suggest that this novel transcutaneous CO$_2$ application might be useful to treat primary tumors, with less side effects, and therefore could be safe for clinical trials.

Acknowledgements

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