Abstract. The present study aimed to evaluate the efficacy of an intra-arterially infused carbon dioxide (CO₂)-saturated solution in sensitizing the anticancer effect of cisplatin in a rabbit VX2 liver tumor model. Forty VX2 liver tumor-bearing Japanese white rabbits were randomly divided into four groups and infused via the proper hepatic artery with a saline solution (control group), CO₂-saturated solution (CO₂ group), cisplatin solution (cisplatin group), or CO₂-saturated solution and cisplatin solution (combined group). The tumor volume (TV) and the relative tumor volume (RTV), RTV = (TV on day 3 or 7)/(TV on day 0) x 100, were calculated using contrast-enhanced computed tomography. Hypoxia-inducible factor-1α (HIF-1α) and carbonic anhydrase IX (CA IX) staining were used to evaluate cellular hypoxia. Cleaved caspase-3 and cleaved caspase-9 were analyzed to assess tumor apoptosis. The mean RTV on days 3 and 7 were 202.6±23.7 and 429.2±94.8% in the control group; 172.2±38.1 and 376.5±61.1% in the CO₂ group; 156.1±15.1 and 269.6±45.2% in the cisplatin group; and 118.3±28.1 and 210.3±55.1% in the combined group. RTV was significantly lower in the CO₂ group than in the control group (day 3; P<0.05), and in the combined group than in the cisplatin group (days 3 and 7; P<0.05). HIF-1α and CA IX suppression, and increased cleaved caspase-3 and cleaved caspase-9 expression, were detected in the CO₂ and combined groups, compared with the other two groups. An intra-arterially infused CO₂-saturated solution inhibits liver VX2 tumor growth and sensitizes the anticancer effect of cisplatin.

Introduction

Solid tumors constitute approximately 90% of all known types of cancer (1). The rapid growth of such tumors alters the cellular microenvironment because of an inadequate oxygen supply and results in hypoxia (2,3). Tumor hypoxia is a potential therapeutic problem because of its adverse impact on the effectiveness of chemotherapy. Carbon dioxide (CO₂) therapy has historically been used for its therapeutic effect on skin problems (4-6). The mechanisms of this beneficial effect are an increase in blood flow and microcirculation, nitric oxide-dependent neocapillary formation, and a partial increase in oxygen pressure in the local tissue, known as the Bohr effect (7). The anticancer effect of the transcutaneous application or intra-arterial infusion of CO₂ has been reported (8-11). In a recent study, the transcutaneous application of CO₂ enhanced the therapeutic efficacy of doxorubicin for human malignant fibrous histiocytoma (12). There is similarly a possibility that the intra-arterial infusion of CO₂ may enhance the therapeutic effect of intra-arterial infusion chemotherapy, but this is unknown.

We evaluated whether the intra-arterial infusion of a CO₂-saturated solution would sensitize the anticancer effect of cisplatin, and we elucidated the mechanism of this therapy in a rabbit VX2 liver tumor model.

Materials and methods

The VX2 liver tumor model. The present study was approved by the Institutional Animal Care and Use Committee (Permission no. P-150202) on February 20, 2015 and was carried out according to the Kobe University Animal Experimentation
Regulations. Forty Japanese white rabbits (age, ~3-4 months old; mean body weight, 2.62±0.03 kg) were implanted with fresh VX2 tumors by injecting 0.1 ml of a VX2 tumor tissue suspension (provided by Japan SLC, Inc., Shizuoka, Japan) into their livers 3 weeks before the intra-arterial infusion. Subsequently, the rabbits were randomly divided into four groups: the control group, the CO2 group, the cisplatin group and the combined group, with 10 in each group. Each material was infused as follows: saline solution into the control group, CO2-saturated solution into the CO2 group, both cisplatin solution and saline solution into the cisplatin group, and both CO2-saturated solution and cisplatin solution into the combined group. For the following procedures, each rabbit was anesthetized with sodium pentobarbital (maximum dose of 50 mg/kg; Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan) via a marginal ear vein.

**Preparation of materials for infusion.** The CO2-saturated solution at pH 4.0 was prepared as previously described (8). The cisplatin solution was prepared by dissolving DDP-H (a fine-powder formulation of cisplatin, IA-call; Nippon Kayaku Co., Ltd., Tokyo, Japan) into the saline solution to a concentration of 1.5 mg/ml. The dose of the CO2-saturated solution and the saline solution was 50 ml each, and the dose of cisplatin was 1.75 mg/kg (as determined from the unpublished data of a DDP-H animal experiment by Nippon Kayaku).

**The CT examination.** Contrast-enhanced CT (Toshiba Activion 16 TXS-031A; Toshiba Medical Systems, Tochigi, Japan or Rx-Ct2; Rigaku, Tokyo, Japan) was performed to evaluate the size of the tumor in the liver. The CT scan was initiated 55 sec after the injection of the contrast medium (Omnipaque 300; Daiichi Sankyo, Tokyo, Japan) at a rate of 0.3 ml/sec via a marginal ear vein. The amount of contrast medium used was set to 2.0 ml/kg. Contrast-enhanced CT was performed before the procedure and 0, 3 and 7 days after the procedure.

**Intra-arterial infusion procedure.** After the CT examination, intra-arterial infusion was performed with a C-arm device (SIREMOBIL Compact L; Siemens Medical Solutions, Erlangen, Germany or ARCADIS Varic; Siemens-Asahi Medical Technologies, Tokyo, Japan). The right femoral artery was exposed and directly punctured with a 22-gauge needle (SURFLO intravenous catheter; Terumo, Tokyo, Japan). A 0.018-inch nitinol guidewire (Cook Medical Japan, Tokyo, Japan) was placed through the needle, and a 4-French introducer catheter (Microintroducer introducer catheter; Cook Medical Japan) was inserted over the guidewire. After the tip of a 2.4 French microcatheter with a swan-neck shape (Nadeshiko; JMS, Co., Ltd., Hiroshima, Japan) was placed into the proper hepatic artery, digital subtraction angiography was performed to confirm the distribution of contrast medium to the liver by manually injecting 1 ml of contrast medium at a rate of 0.1 ml/s (Fig. 1). Each material of the group, as described above, was infused after the angiography. The CO2-saturated solution and the saline solution were infused for 10 min, and the cisplatin solution was administered for 3 min through the catheter. After the injection of the solution, the catheter was removed, and the right femoral artery was ligated to achieve hemostasis. The incision wound was sutured, and the rabbits were observed for 7 days while maintaining a normal feeding regimen. All rabbits were euthanized after CT scanning on day 7, and the liver of each rabbit was carefully excised and processed for histological examination.

**Tumor growth and volume measurement.** All recorded CT volumetric data were transferred to Ziostation software (Ziosoft, Inc., Tokyo, Japan) and reconstructed in 3-mm thick slices. Two experienced radiologists, who were blinded to the treatment group status, manually traced the contour of the VX2 tumor area in each slice. All measurements were independently performed twice, and the tumor area was determined as the mean value of all measurements. The tumor volume (TV) was then calculated using the following formula: \( \text{TV} = \frac{\text{total circumscribed area in each slice} \times \text{CT section thickness}}{\text{mean body weight in kg}} \). We evaluated whether the therapeutic effect of the combined group was antagonistic, additive or synergistic by comparing with expected RTV for additive effect. Expected RTV for additive effect was calculated based on the following formula: expected RTV for additive effect = (RTV of the CO2 group) x (RTV of the cisplatin group)/(RTV of the control group), as reported (13).

**Histology.** Liver tissue was fixed in a 10% phosphate-buffered formaldehyde solution, and 7-mm sections were obtained and embedded in paraffin. Serial sections were then cut at 6-µm thickness. One section was stained with hematoxylin and eosin (H&E) and contiguous sections were immunofluorescently stained using 4',6-diamidino-2-phenylindole (DAPI) stain and APO-Direct kit (Bay Bioscience, Co., Ltd., Kobe, Japan) to evaluate DNA fragmentation. Immunofluorescence assay of DNA fragmentation is described below. All histopathologic specimens were evaluated by a pathologist under a light microscope (Keyence Corp., Osaka, Japan) and the apoptotic area was described, based on the fluorescence staining results.
Immunoblot analysis was performed to evaluate the apoptotic pathway of caspase-3 and caspase-9. Tumor lysates were prepared from tumor tissues in whole-cell lysis buffer (Mammalian Protein Extraction Reagent; Thermo Fisher Scientific, Rockford, IL, USA). Samples were processed with standard western immunoblotting procedures. Membranes were incubated overnight at 4°C with the following antibodies in Can Get Signal immunoblotting solution (1:1,000). Membranes were incubated with the appropriate secondary antibody conjugated goat anti-mouse IgG polyclonal antibody (Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature. The signal was developed as a brown reaction product using the peroxidase substrate mino analyzer (LAS-3000 mini; Fujifilm, Tokyo, Japan) was used to detect signals.

Statistical analysis. Statistical analyses were conducted using JMP 12.0.1 (SAS Institute, Inc., Cary, NC, USA). The data are presented as the mean ± standard deviation, unless indicated otherwise. The significance of differences between groups was evaluated using the two-tailed Student’s t-test, and by one-way analysis of variance with post-hoc Tukey’s honestly significant difference test for multiple comparisons. P<0.05 was considered significant.

Results

All procedures were performed successfully, and all rabbits survived for 1 week after the procedure. The rabbits were euthanized on day 7, and the liver of each rabbit was excised and processed for histological examination. The mean body weight on the procedure day and on day 7 were, respectively, 2.70±0.23 kg and 2.76±0.18 kg in the control group; 2.66±0.23 and 2.64±0.16 kg in the cisplatin group; and 2.58±0.08 and 2.59±0.07 kg in the combined group. There were no significant differences among the four groups (P>0.05).
RTV is shown in Fig. 3. The mean RTV of the CO\textsubscript{2} group on day 3 was significantly decreased, compared with the control group (P<0.05), but there was no significant difference on day 7 (P=0.16). RTV on days 3 and 7 was significantly lower in the combined group than in the cisplatin group (P<0.05). The mean RTV on day 7 was also significantly lower than expected RTV for additive effect (P<0.05). RTV was calculated based on the following formula: RTV = (TV on day 3 or 7)/(TV on day 0) x 100. The mean tumor volumes and RTV are also presented. CO\textsubscript{2}, carbon dioxide; NS, not significant. *P<0.05.

Evaluation of apoptosis. Representative H&E-stained liver sections demonstrated an increased apoptotic area and decreased tumor area in the CO\textsubscript{2} and cisplatin groups, and more so in the combined group, compared with the control group. The scale bars indicate 1,000 µm. CO\textsubscript{2}, carbon dioxide.

Evaluation of hypoxia. HIF-1α and CA IX staining demonstrated suppression of HIF-1α and CA IX in the CO\textsubscript{2} and combined group (Fig. 6A and 6B). As for the quantification of HIF-1α expression, the numbers of hypoxic cells per area were 246±118 in the control group, 21±10.4 in the CO\textsubscript{2} group, 152±40.7 in the cisplatin group and 16±7.3 in the combined group. Hypoxic cells were significantly more in the control and cisplatin groups compared with the CO\textsubscript{2} and combined groups (Fig. 6C and 6D; P<0.05).

Discussion

There was a significant difference in tumor growth between the control group and the CO\textsubscript{2} group, and between the cisplatin group and the combined group. The intra-arterial infusion of the CO\textsubscript{2}-saturated solution inhibited tumor growth and
sensitized the anticancer effect of cisplatin. The results of this study will contribute to improving the therapeutic effect of intra-arterial chemotherapy using cisplatin.

CO₂ effect of improving hypoxia and inducing apoptosis have been explained by some mechanisms. The first mechanism is direct antitumor effect of CO₂. There are several reports showing that intracellular calcium (i.e., Ca²⁺) concentrations increased by CO₂ induces the expression of peroxisome proliferator-activated receptor gamma coactivator-1 alpha and mitochondrial biogenesis (14-19). In a human malignant fibrous hystiocytoma tumor model, transcutaneous CO₂ treatment increased intracellular Ca²⁺ concentrations and induced mitochondrial DNA apoptosis (12). In vitro study of a human neuroblastoma cell model, intracellular reactive oxygen species induced by CO₂ intracellular reactive oxygen species, lead to proapoptotic p53 signal stimulation, DNA damage, and cell death through the mitochondrial pathway (20). The second and third possible mechanisms are related to oxygenation and pH in the tumor microenvironment. The present study did not show evidence of increased partial pressure of oxygen or oxygen saturation and decreased pH in VX2 tumor tissue during the procedure. However, a previous study (8) demonstrated that transcutaneous CO₂ application significantly lowers intracellular pH, decreases oxyhemoglobin, and increases deoxyhemoglobin in treated muscle.

CO₂ therapy is considered to improve tumor hypoxia and induce the mitochondrial pathway of apoptosis as described above. Moreover, this therapy was reported to suppress vascular endothelial growth factor and HIF-1α (21). In this study, there was less HIF-1α expression and more cleaved caspase-3 and caspase-9 expression in the CO₂ and combined groups than in the control and cisplatin groups. This result revealed that intra-arterial CO₂ infusion could improve hypoxia and induce apoptosis in tumors. Caspase-3 is activated in apoptotic cells by the extrinsic (i.e., death ligand) and intrinsic (i.e., mitochondrial) pathways and caspase-9 reflects mitochondrial apoptosis. Cleaved caspase-3 and cleaved caspase-9 are activated forms of caspase-3 and caspase-9, and are commonly used to detect apoptosis (22). HIF-1α is a basic helix-loop-helix-PAS (bHLH-PAS) transcription factor that has an essential role in O₂ homeostasis (6,7,9,10), and has recently emerged as a major factor influencing tumor proliferation and malignant progression (23,24). Hypercapnia was reported to counter-regulate the activation of the HIF pathway by reducing the intracellular pH (25).
Minimizing hypoxia and suppression of HIF-1α expression in tumors also has the potential to enhance chemotherapeutic effects (26). In this study, the combined group achieved a higher tumor growth inhibition rate, compared with the other groups and expected additive effect. We expected that a CO2-saturated solution would sensitize the tumor to the anticancer effect of cisplatin by suppressing HIF-1α expression. Previous reports support our hypothesis: Ai et al (27) revealed that the genetic knockdown of HIF-1α or pharmacological promotion of HIF-1α degradation enhanced the response of ovarian cancer cells to cisplatin, and diindolylmethane is reportedly a cisplatin sensitizer that exerts its effect by targeting signal transducer and activator of transcription 3, which suppresses HIF-1α and vascular endothelial growth factor (28).

Intra-arterial infusion of CO2 is well-known to interventional radiologists as a negative contrast medium. CO2 has advantages over other treatments, such as its lack of nephrotoxic and allergenic effects on the human body. Moreover, it is markedly less expensive than other drugs (the typical cost for CO2 is 3 cents per 100 ml) (29). Thus, we believe that a CO2-saturated solution is the ideal material for sensitizing the anticancer solution would sensitize the tumor to the antitumor effect of cisplatin by suppressing HIF-1α expression in tumors also has the potential to enhance chemotherapeutic effects (26).

In conclusions, intra-arterial infusion of a CO2-saturated solution as a cisplatin sensitizer should inhibit tumor growth and sensitize the anticancer effect of cisplatin by suppressing HIF-1α expression in a rabbit VX2 liver tumor model.

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