Effect of carbon dioxide pneumoperitoneum on human renal cell carcinoma proliferation and metastasis in an orthotropic xenograft nude mouse model

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Abstract

Introduction: This study aimed to explore the effect of carbon dioxide (CO₂) pneumoperitoneum on tumor proliferation and metastasis in an orthotropic xenograft nude mice model of human renal cell carcinoma (RCC) and evaluate the safety of CO₂ pneumoperitoneum laparoscopy for treating RCC.

Material and methods: RCC 786-0 cells were injected to establish an orthotropic xenograft model. Fifty nude mice were given orthotropic inoculations and randomized to five groups: group A (control); group B (CO₂ pneumoperitoneum for 2 h); group C (CO₂ pneumoperitoneum for 4 h); group D (CO₂ pneumoperitoneum for 4 h and 24 h after waking); group E (CO₂ pneumoperitoneum for 4 h and 48 h after waking). The proliferation status was observed in RCC specimens by immunohistochemical staining for Ki67. The protein levels of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) were examined by western blotting.

Results: All groups showed similar Ki67-positive staining in RCC samples (p > 0.05). The relative expression of HIF-1α and VEGF gradually increased in both group B and group C, as compared with group A, but only the difference between group C and group A reached statistical significance (p < 0.05). The protein levels of HIF-1α and VEGF decreased in both group D and group E, as compared with group B and group C; however, the differences between group D, group E, and group A did not reach statistical significance (p > 0.05).

Conclusions: In an orthotropic xenograft nude mice model of RCC, CO₂ pneumoperitoneum has no effect on expression of the cellular proliferation marker Ki67. However, CO₂ pneumoperitoneum rapidly induces transient expression of HIF-1α and VEGF. Thus, CO₂ pneumoperitoneum laparoscopy may be a safe method for treating RCC.

Key words: carbon dioxide pneumoperitoneum, renal cell carcinoma, tumor proliferation, hypoxia-inducible factor-1α, vascular endothelial growth factor.

Introduction

While several studies have investigated the effects of simulated CO₂ pneumoperitoneum on tumor proliferation- and metastasis-related factors in vitro and in vivo [1–11], none have explored the effects in an orthotropic xenograft nude mice model of renal cell carcinoma (RCC). In RCC, many molecules play key roles in tumor proliferation and metastasis via inducing endothelial cell hyperplasia and degradation of extracellular matrix, such as hypoxia-inducible factor-1α (HIF-1α), cyclooxygenase
(COX), heparanase (HPA), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs) [12], vascular cell adhesion molecule 1 (VCAM-1) and E-cadherin. The abdominal cavity can be an oxygen-rich environment under normal physiological conditions. Since pneumoperitoneum with 100% CO₂ has been used during laparoscopy, it is necessary to study the effect of the induced hypoxic environment on local tissue during the surgical procedure. Ridgway et al. reported that the initial hypoxia during laparoscopic surgery was the most likely mechanism mediating a tumor’s invasive tendencies [13]. Under anoxic conditions, HIF-1 is a critical regulator of various transcriptional responses, directly binding to multiple target genes in order to mediate adaptive responses to hypoxia. In malignant tumors, HIF-1 is known to play a key role in maintaining energy metabolism, promoting angiogenesis, and inducing proliferation and metastases [14–16]. A main subunit of HIF-1, HIF-1α, has been defined as a central mediator of RCC tumorigenesis [17]. In hypoxic tissues, HIF-1α drives transcription of VEGF, which is one of the most important growth factors responsible for neovascularization. The process of tumor angiogenesis also plays key roles in tumor proliferation, invasion, and metastasis [18]. The Ki67 protein is a well-established cellular marker of proliferation, and the reflected tumor proliferative rate is related to tumor initiation, progression, metastases, and prognosis [19].

Thus, we examined HIF-1α, VEGF and Ki67 expression to explore the mechanism of CO₂ influence on tumor proliferation and metastasis. In addition, the study was conducted to evaluate the safety of CO₂ pneumoperitoneum laparoscopy for treating RCC.

Material and methods

The RCC cell line 786-O was obtained from the Shanghai Institutes for Biological Sciences, Institute of Biochemistry and Cell Biology at the Chinese Academy of Science Institute. Seventy BALB/c nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The surgery department of our hospital provided the surgical CO₂ insufflator (Olympus). The Ki67 cellular proliferation detection kit for immunohistochemical (IHC) staining was obtained from Nanjing KeyGen Biotech Co., Ltd. Primary antibodies against human HIF-1α and VEGF were purchased from Santa Cruz Biotechnology. The experimental procedures and the animal use and care protocols were approved by the Committee on Ethical Use of Animals of Shanghai Tenth People’s Hospital.

The 786-O cells (1 × 10⁷/ml) were suspended in RPMI 1640 medium without serum. The nude mice were anesthetized with a single intraperitoneal (i.p.) injection of 1% pentobarbital sodium (50 μl/10 g). A small vertical incision was made through the dorsal muscles of the body wall in the region of the left kidney. Cells (0.1 ml/mice) were injected into the renal capsule, and the muscles and skin were closed with silk sutures. Ten weeks later, tumors were removed for analysis. Three mice had died during the inoculation and two mice died during the tumor growth period. Finally, 50 tumor-implanted mice were individually tagged and randomized into control and test groups designated as group A, B, C, D and E consisting of 10 mice each.

In group A, the mice were anesthetized with a single i.p. injection of 1% pentobarbital sodium (50 μl/10 g) for 4 h and sacrificed by cervical dislocation. Each tumor was resected and divided into two parts, one of which was fixed with 4% paraformaldehyde in liquid nitrogen. In groups B, C, D and E, the mice were anesthetized and a size 7 needle for the scalp vein was used as the Veress needle and inserted from the left side of the back to create the pneumoperitoneum. The needle was connected to the surgical CO₂ insufflator by a plastic tube. Carbon dioxide pneumoperitoneum pressure was set to 15 mm Hg, CO₂ flow to 60 ml/min and temperature to 37°C through the insufflator, after which 100% CO₂ insufflations were applied to the groups for 2 h, 4 h, 4 h, or 4 h, respectively. During this procedure, anesthesia was maintained by i.p. injection of 1% pentobarbital sodium. Mice from groups D and E were allowed to recover under normal conditions for 24 h and 48 h after waking, respectively, and then sacrificed by cervical spine dislocation. All tumor samples from groups D and E were divided into two parts for fixing with 4% paraformaldehyde and storing in liquid nitrogen, respectively. During this process, one mouse in group C died during the CO₂ insufflation and one mouse in group D died of unknown causes during the recovery period.

Sections of the 4% formaldehyde-fixed paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H + E) to evaluate the RCC and cell pleomorphism. One section was subjected to IHC staining of Ki67. After visualization by DAB substrate, microscopic images were captured and analyzed by the Leica Automatic Microscope and Image Analysis System. All data were calculated as mean and standard deviation (SD). Larger optical density (OD) values indicate stronger positivity, which reflects the proliferation state of a cell.

Relative expression of HIF-1α and VEGF in tumor specimens was determined by western blotting. After estimating protein concentration by Coomassie Blue G250, 30 μg of total protein was loaded and run on a 10% SDS-PAGE gel at 80 V for
30 min, after which the voltage was increased to 100 V and the gel run until the bromophenol blue tracking dye reached the bottom. After transferring to nitrocellulose membranes at 120 V for 90 min, the membranes were blocked with 2% bovine serum albumin overnight and washed with Tris-buffered saline (TBS; 10 mmol/l, pH 7.4). The appropriate primary and secondary antibodies were then added and the membranes incubated for 2 h. After washing with TBS, the immunoreactive bands were detected with chemiluminescence reagents, scanned by the Bio-Rad Gel Imaging System, and quantified by the accompanying Quantity One software. The relative expression level was calculated as the gray value of target bands divided by the gray value of the GAPDH band as an internal control.

**Statistical analysis**

All data were analyzed by SPSS 13.0 software. Significance of intergroup differences for the OD values of IHC staining and the relative protein expression was assessed by the ANOVA test. Values of $p < 0.05$ were considered significant.

**Results**

**Tumor-implanted mice**

Obvious round- or oval-shaped tumors had formed around the left kidney by 10 weeks after inoculation. Tumors ranged in size from 0.5 cm to 2 cm in diameter (1.3 ±0.4 cm). Finally, 52 of 70 mice with a tumor burden survived the inoculation procedure and tumor growth period without developing dyscrasia. The overall tumor formation rate was 74.3%. Renal parenchyma and capsule adhesion with tumor tissues and intra-abdominal metastasis were observed in 11 of the inoculated mice.

**H + E-stained sections and Ki67-positive expression**

H + E staining showed obvious cell pleomorphism in tumor tissues and typical morphological features of RCC. Tumor samples from all groups showed positive staining for Ki67 expression (Figure 1). The OD values are listed in Table I. There was no significant difference among the mean OD values for any of the groups ($p > 0.05$).

**Western blotting analysis of HIF-1α and VEGF expression**

The quantification of the relative expression of HIF-1α and VEGF is presented in Figure 2. The expression of both HIF-1α and VEGF was gradually increased in groups B and C, as compared with group A. While the difference between group A and group B did not reach statistical significance ($p > 0.05$), the difference between group C and group A did ($p < 0.05$). Compared with groups B and C, groups D and E showed lower levels of HIF-1α and VEGF expression. However, the levels in groups D and E were not significantly different from those in group A ($p > 0.05$).

**Discussion**

The safety of laparoscopy for treating malignancies remains controversial, especially in relation to whether the laparoscopic procedure itself
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Can stimulate growth and/or induce metastasis of tumor cells. It has been reported that the laparoscopic CO2 pneumoperitoneum procedure can increase both abdominal pressure and hypoxia, as well as decrease the pH in the abdominal cavity [20]. Recent research has explored the potential effects of CO2 pneumoperitoneum on tumor metastasis and metastases during laparoscopy. The recent studies on CO2 pneumoperitoneum can mainly be divided into two groups: the first has investigated the effects of different CO2 pneumoperitoneum parameters on cancer cells’ behavior using in vitro systems, while the second has evaluated the impact of various CO2 pneumoperitoneum parameters in vivo using i.p. injection or xenograft tumor models. However, the ability of CO2 insufflations during laparoscopy to induce or inhibit tumor growth and metastases remains controversial [1–11].

The i.p. xenograft models, and not the orthotopic transplant tumor models, used in most of the animal studies were significantly different from the clinical tumor growth environments. In addition, very few studies have focused on the effect of CO2 pneumoperitoneum on RCC. Therefore, we designed the current study using an orthotopic xenograft nude mice model, which more closely mimics the clinical tumor environment. Our study also aimed to recapitulate the clinical CO2 pneumoperitoneum environment for treating RCC to the utmost extent. First, we simulated the RCC growth environment using a human RCC orthotopic xenograft. Second, two time points (2 h and 4 h) were examined, because most laparoscopic procedures should be completed within 4 h; for example, laparoscopic radical or partial nephrectomies for RCC should be finished within 2 h. Third, CO2 insufflations at 15 mm Hg pressure were used, since this is consistent with the common pressure used in the clinic.

At the cellular and tissue levels, there were no significant differences in Ki67 expression of the control and experimental groups of our study. At the protein level, 2 h of CO2 pneumoperitoneum increased HIF-1α and VEGF (vs. control group, p > 0.05), in agreement with previous results. Cai et al. [21] reported that VEGF expression was significantly increased after 1 h of CO2 pneumoperitoneum.

### Table I. OD values of Ki67 immunohistochemical staining

| Characteristic | OD values | Group A (n = 10) | Group B (n = 10) | Group C (n = 9)* | Group D (n = 9)* | Group E (n = 10) |
|---------------|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|
|               |           | 1.2224          | 1.2151          | 1.2079          | 1.2732          | 1.2761          |
|               |           | 1.3190          | 1.2605          | 1.2351          | 1.2705          | 1.1864          |
|               |           | 1.1889          | 1.2715          | 1.1906          | 1.2606          | 1.2673          |
|               |           | 1.2605          | 1.2602          | 1.1433          | 1.3015          | 1.2961          |
|               |           | 1.2231          | 1.2723          | 1.2977          | 1.2051          | 1.2316          |
|               |           | 1.2452          | 1.1817          | 1.2854          | 1.2320          | 1.1943          |
|               |           | 1.2427          | 1.2674          | 1.1823          | 1.2302          | 1.2322          |
|               |           | 1.1932          | 1.2841          | 1.2696          | 1.1557          | 1.2015          |
|               |           | 1.2086          | 1.2006          | 1.2205          | 1.2461          | 1.1315          |
|               |           | 1.2524          | 1.1618          |                 |                 |                 |

Mean ± SD 1.2356 ±0.0381 1.2375 ±0.0435 1.2258 ±0.0512 1.2416 ±0.0429 1.2313 ±0.0537

Value of p** – 0.93 0.65 0.78 0.84

*One mouse in group C died during the CO2 insufflation and one mouse in group D died of unknown causes during the recovery period.

**Comparison between group A and groups B, C, D, E, between all groups, p = 0.959

![Figure 2. Relative protein expression of HIF-1α (black) and VEGF (grey) in all groups](image)

C p < 0.05 and B, D, E p > 0.05 vs. group A

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peritoneum. In our RCC orthotropic xenograft model, with tumors located in the kidney where sufficient blood supply was available, the overall impact of pneumoperitoneum pressure on the tumor cell environment was expected to be less than that of an in vitro or i.p. injection model. The group administered 4 h of CO₂ pneumoperitoneum showed significantly increased HIF-1α and VEGF (vs. control group, \( p < 0.01 \)). However, the induced HIF-1α and VEGF expression gradually decreased during the 24 h and 48 h recovery (vs. control group, \( p > 0.05 \)). Our results might reflect the normal physiological mechanisms of nude mice, in which CO₂ pneumoperitoneum has no effect on tumor tissues with lower expression of HIF-1α and VEGF. Thus, the effect of CO₂ pneumoperitoneum on the changes of tumor proliferation and metastasis may be reversible. Zhang et al. [22] reported similar results, in that several hours of hypoxia caused obvious changes in expression of proteins related to tumor metastasis that gradually returned to baseline levels as the normoxic environment was reestablished. However, the different effects of transiently raising HIF-1 and VEGF on RCC proliferation and metastasis should not be ignored. HIF-1α was reported to induce apoptosis of solid cancer cells by the p53 pathway [23, 24], whereas VEGF-D expression was correlated with the depth of tumor invasion and lymph node metastasis in human colorectal carcinoma [25]. The different effects of different pressures and concentrations for CO₂ insufflation on RCC proliferation and metastasis should be noted. Bourdel et al. [26] reported that postoperative peritoneal dissemination of ovarian cancer cells is not promoted by a CO₂ pneumoperitoneum with 2 mm Hg intraperitoneal pressure in a mouse model when assessed on postoperative day 14. Tan reported that the short-term effect of CO₂ on transitional-cell carcinoma growth and apoptosis is complex, and high CO₂ concentrations (> 5%) and high insufflation pressures (≥ 15 mm Hg) are most effective in reducing tumor-cell adhesion and growth [27].

In the present study, we mimicked CO₂ pneumoperitoneum environments for treating RCC using an orthotropic xenograft model. At the cellular and tissue levels, CO₂ pneumoperitoneum had no effect on RCC proliferation. At the protein level, CO₂ pneumoperitoneum had a reversible effect on proteins related to renal cancer cell proliferation and metastasis.

Our study had two distinct limitations. We did not observe the growth rate of tumors in live mice which underwent creation of CO₂ pneumoperitoneum vs those that did not. It was reported that renal cancer growth could be enhanced by pneumoperitoneum vs gasless procedure in rats [28]. Therefore, the growth rate of tumors in animal models which underwent creation of CO₂ pneumoperitoneum needs to be further confirmed. The mouse model of pneumoperitoneum peritoneal inoculation of human line RCC is one of the possible ways of assessing safety of surgical treatment in humans, but it is not a typical human appearance of RCC, which is rather retroperitoneal and intrarenal. We did not design our study to investigate all these issues, so the relevant study needs to be conducted in future research.

In conclusion, in a human RCC orthotropic xenograft mouse model, CO₂ pneumoperitoneum has no effect on the Ki67 cell proliferation marker. However, CO₂ pneumoperitoneum rapidly induces HIF-1α and VEGF expression, but these changes can be reversed upon resolution of hypoxia. Our results suggest that CO₂ pneumoperitoneum laparoscopy may be a safe method for treating RCC.

Acknowledgments
Yuan-Zhuo Chen and Yun-Fei Xu — contributed equally to this work.

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