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• BRIEF REPORTS •

Effects of carbon dioxide and nitrogen on adhesive growth and expressions of E-cadherin and VEGF of human colon cancer cell CCL-228

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Abstract

AIM: To study the effects of carbon dioxide on the metastatic capability of cancer cells, and to compare them with that of nitrogen.

METHODS: The colon cancer cell CCL-228 was treated with 100 % carbon dioxide or nitrogen at different time points and then cultured under normal condition. Twelve hours after the treatment, the survival rates of suspension cells and the expressions of e-cadherin and VEGF were examined.

RESULTS: After 60 min of carbon dioxide and longer time of nitrogen treatment, the suspended cells increased and the expression of e-cadherin decreased while the expression of VEGF was enhanced significantly. And the effects of nitrogen were similar to, but weaker than, those of carbon dioxide.

CONCLUSION: Carbon dioxide may improve the metastatic capability of cancer cells and its effects are significantly stronger than that of nitrogen. A sequential use of carbon dioxide and nitrogen in pneumoperitoneum may take the advantage of both gases.

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INTRODUCTION

The indications for laparoscopic surgery have expanded significantly thanks to the improved expertise and equipment. The main method to expose the operative field is carbon dioxide pneumoperitoneum. A large number of laparoscopic surgeries for gastroenteric malignant diseases, especially rectum or colon resections, have been performed. But there is a dispute on whether laparoscopic surgery is suitable for malignant diseases[1-6]. To investigate if carbon dioxide influences the metastatic capability of gastroenteric cancer cells, we studied the effects of carbon dioxide on the adhesive growth and the expression of cadherin and VEGF of a colon cancer cell line CCL-228 propagated *in vitro*. And the effects of carbon dioxide were compared with those of nitrogen. We found that, the metastatic ability of CCL-228 cells might elevate after carbon dioxide treatment and the influence of nitrogen was significantly milder than that of carbon dioxide. Based on these results, we proposed a sequential pneumoperitoneum method to reduce the side-effect but remain the safe-guard ness of carbon dioxide, *i.e.*, to establish the pneumoperitoneum with carbon dioxide and to maintain it with nitrogen, and before the end of the surgery, insufflating carbon dioxide to remove the unabsorbable nitrogen. In the present study, we evaluated the influence of the sequential gas treatment on the expression of E-cadherin and VEGF in CCL-228 cells.

MATERIALS AND METHODS

Cell culture

Human colon cancer cell line CCL-228, supplied by the Type Culture Collection Center, Wuhan University, was maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum. The cell cultures were maintained as monolayer in a plastic flask and incubated in 5 % CO_2, 95 % air at 37 ℃. The cultures were free of mycoplasma.

Carbon dioxide or nitrogen treatment

The CCL-228 cells were seeded onto 6-well plates (1×10^6 cells per well). When the button was 80 % covered, the cells were divided into two groups. Three parallel wells of cells in each group were incubated either in 100 % CO_2 (CO_2 group) or in 95 % N_2+5 % CO_2 (N_2 group) for 30, 60, 120, 180 min, respectively. To evaluate the effect of sequential pneumoperitoneum on the metastatic ability of colon cancer cells, 3 wells of CCL-228 cells were treated with CO_2 for 15 min, with nitrogen for 90 min or 150 min, and then with CO_2 again for 15 min. After the gas treatment, all cells were incubated again in 5 % CO_2, 95 % air for 12 h.

Histology

The supernate was collected and stained with typan blue, and the survival rate of the supernatant cells was estimated.

Immunohistochemistry

The adhesive growth cells were collected and stained for immunohistochemical studies on the expressions of VEGF and E-cadherin. The cell smear was fixed with cold acetone for 10 min at room temperature and rinsed with phosphate-buffered saline (PBS). The endogenous peroxidase was blocked using 3 % hydrogen peroxide for 10 min and the unspecific combined site was blocked with normal goat serum. Excessive blocking serum was drained and the samples were incubated at 40 ℃ for 18 h with the appropriate dilution of monoclonal mouse anti-human

VEGF or E-cadherin (Santa Cluz). Each sample was then rinsed with PBS and incubated for 10 min at room temperature with 50 μl of biotin-labeled goat anti-mouse IgG. The samples were then rinsed with PBS and incubated for 10 min at room temperature with 50 μl peroxidase conjugated avidin. The smears were rinsed with PBS and incubated for 5 min with diaminobenzidine. They were then washed with water and counterstained with Mayer' s hematoxylin and fixed with neutral resin. The smears were examined under microscope and positive reaction was indicated by brownish precipitate in cytoplasm.

Analysis of results

The smears were analyzed with MPZAS-500 multimedia color pathological graph analyzing system. The average integral light density (ILD) of positive staining in each sample was obtained and presented as $\bar{x}\pm s$. Results were then analyzed with Student' s *t* test.

RESULTS

Effects of CO_2 and N_2 on adhesive growth of CCL-228

Sixty min of CO_2 treatment or 120 min of N_2 treatment was sufficient to increase the rate of suspending growth cells very significantly (Table 1). The difference of rates of alive suspending cells between CO_2 and N_2 treatments was very significant after 60 min of treatment ($P<0.01$).

Table 1 Counts and survival rate of suspension CCl-228 cells after CO_2 or N_2 treatment

Duration	Counts of suspension cells (×1000 / ml)		Survival rate of suspension cells	
	CO_2 group	N_2 group	CO_2 group	N_2 group
0 min	4.2±0.1		0.884±0.011	
30 min	7.5±2.9	4.9±2.5	0.912±0.018	0.852±0.022
60 min	16.7±5.4[ab]	5.2±0.7	0.914±0.041	0.897±0.021
120 min	23.2±10.7[ac]	8.5±1.4[d]	0.904±0.034	0.912±0.023
180 min	32.1±5.5[ac]	9.7±3.3[d]	0.845±0.028	0.912±0.032

[a]$P<0.01$, *vs* the 0 min in same group; [b]$P<0.05$, *vs* N_2 group at same time; [c]$P<0.01$, *vs* N_2 group at same time; [d]$P<0.05$, *vs* 0 min in same group.

Effects of CO_2 and N_2 on expression of E-cadherin in CCL-228 cell

Both CO_2 and N_2 treatment depressed the expression of E-cadherin in CCL-228 cell (Figure 1). The expression of E-cadherin after 60 min or longer time of CO_2 treatment was significantly different from that after the same time periods of N_2 treatment. Comparing with the expression of E-cadherin before any gas treatment, 120 min or longer time of CO_2 treatment produced a very significant depression ($P<0.01$) and the same periods of N_2 treatment caused a significant depression ($P<0.05$).

Effects of CO_2 and N_2 on expression of VEGF in CCL-228 cell

Both CO_2 and N_2 treatment improved the expression of VEGF in CCL-228 cell (Figure 2). Thirty min to 120 min of CO_2 treatment caused significantly higher expression of VEGF comparing with the normal control and the same periods of N_2 treatment ($P<0.05$). CO_2 treatment for 180 min resulted in a VEGF expression significantly lower than CO_2 treatment for 120 min, but still significantly higher than normal control. As for N_2, 30 min to 180 min of treatment resulted in a continuous increase of VEGF expression.

Effects of sequential treatment with CO_2 and N_2 on expression of E-cadherin and VEGF in CCL-228 cell

The effects of sequential gas treatment for 120 min or 180 min on expression of E-cadherin and VEGF in CCL-228 cell were not significantly different from those of continuous nitrogen treatment but significantly milder than those of continuous carbon dioxide treatment (Figures 1 and 2).

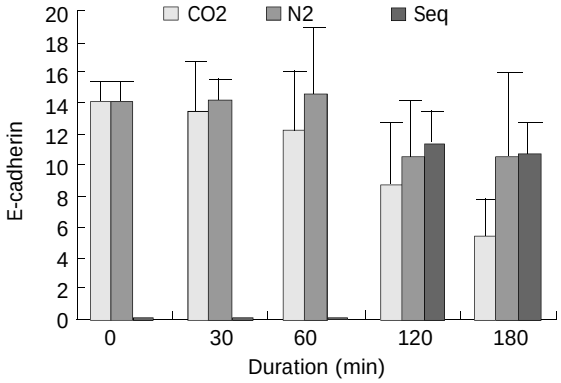

Figure 1 The expression of E-Cadherin in CCL-228 cells after gas treatment.

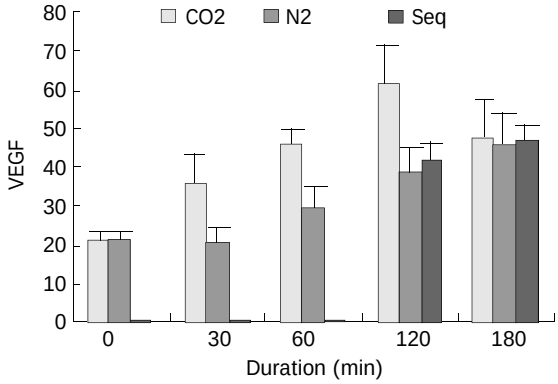

Figure 2 The expression of VEGF in CCL-228 cells after gas treatment.

DISCUSSION

It has been verified that laparoscopic surgery has many virtues such as minimal injury, less pain, sooner rehabilitation and less intervention in the immune function[7-11]. Now many gastroenteric surgeries including cancer resection could be fulfilled under laparoscope. Clinical trials have proved that laparoscopic surgery is feasible for gastroenteric malignant diseases. However, cancer metastasis in trocar site has been reported and has aroused a debate about whether laparoscopic surgery is suitable for malignant diseases. There is no consensus on if the incidence of metastasis in trocar site was accurately higher than that in the wound after open surgery[6], because the cases were few in each paper and there has been a decline in incidence of trocar site metastasis in recent reports, due to more attention paid to the wound protection during extraction of the specimen. Moreover, open surgery has been reported to increase cancer metastasis[12-15], and had more intervention in immunity. Therefore, whether laparoscopic surgery is suitable to early malignant diseases needs to be clarified by more clinical trials and further laboratory studies. In the present experiment, we studied the effect of carbon dioxide on the adhesive ness of colon cancer cells to the culture plate and the expressions of E-cadherin and VEGF. It should be noted that, the duration of carbon dioxide treatment was much longer than others because a laparoscopic surgery for gastroenteric cancer resection often lasts 2-3 hours.

We found that both carbon dioxide and nitrogen influenced the adhesive growth of CCL-228 cells. The cells in supernate increased after CO_2 or N_2 treatment. The rate of living cells in

supernate had no significant change confirmed by typan blue staining. Therefore, increase of cells in the supernate was a result of detachment of cells from the culture plate, but not the result of apoptosis or necrosis after gas treatment. It is suggested that CO_2 or N_2 could influence the adhesive ness of CCL-228 to matrix, resulting in easier metastasis by improving the detachment of cancer cells from the original lesion. We found that 30 min of CO_2 treatment caused an obvious increase of suspensive growth but the increase was not statistically significant, while 60 min and longer time produced very significant results. There were several laboratory reports on the effect of CO_2 on liver metastasis of intestinal cancer but the conclusions were controversial. We suppose that the controversy might result from a relatively short duration of pneumoperitoneum. Considering that laparoscopic surgery for malignant diseases usually needs several hours, the pneumoperitoneum time in experiments should be long enough to produce a practical result.

E-cadherin is one of the key adhesive molecules to mediate intercellular adhesion. It has been proved that, E-cadherin expression is depressed in metastatic tissues, which is helpful for the detachment of cancer cells from original lesions[16-20]. We examined the expression of E-cadherin in CCL-228 to explore if it plays a role in the influence of carbon dioxide on the adhesive growth of CCL-228. The results showed that, carbon dioxide treatment depressed E-cadherin expression and the effect was positively related to the duration of carbon dioxide treatment. Because cancer metastasis course includes the detachment of cells from the original lesion and also the settlement in the new environment, depression of adhesion has a two-edged effect on the metastasis of cancer. It is helpful for cancer cell dissemination but unfavorable to the settlement of circulating cancer cells.

The expression of VEGF is essential for cancer metastasis because it is a vital factor in tumor angiogenesis. A great deal of researches have indicated that cancer cells with higher VEGF expression results in much more persistently growing metastatic lesions, and inhibition of VEGF expression or function could interrupt the metastasis[21-25]. Therefore, we studied further the effect of carbon dioxide on VEGF expression in CCL-228 and found that it promoted VEGF expression. The promotion of VEGF expression suggests that decrease of E-cadherin expression in CCL-228 cells was not due to a general inhibition of protein synthesis by carbon dioxide, and the results implicate that carbon dioxide pneumoperitoneum was favorable for not only the detachment of cancer cells from the primary lesion but also for the growth of micrometastatic cells and their aggregation into a mass.

Many factors are supposed to be associated with the favorable effect on cancer growth and metastasis by carbon dioxide. The topical acidosis might be the main reason, and the less absorbable gases such as nitrogen and helium have less influence on metastasis[1,26,27]. In this experiment, we found that, comparing with carbon dioxide, nitrogen had a similar but much more mild effects on cancer cells. Since carbon dioxide had a significant duration-effect relationship with the expression of E-cadherin and VEGF, reduced carbon dioxide insufflation duration might be less harmful to patients with malignant diseases. Here we propose a sequential pneumoperitoneum, namely, establishing the pneumoperitoneum with carbon dioxide and maintaining it with nitrogen, and then, before the end of surgery, insufflating carbon dioxide to remove the unabsorbable nitrogen. We found that, during the 120 min or 180 min of gas treatment, when carbon dioxide was applied in the first and last 15 min but replaced with nitrogen in the time between E-cadherin and VEGF expressions were significantly different from that by persistent carbon dioxide treatment. Therefore, we think that in laparoscopic surgery for malignant diseases, the sequential pneumoperitoneum method may take the advantages of both nitrogen and carbon dioxide, as the effect of carbon dioxide on metastasis is reduced by nitrogen replacement during the operation, and the safe guard ness of carbon dioxide to prevent from gas embolism, usually happening during the establishment of pneumoperitoneum, is remained. And the remaining gas in abdomen after surgery is also carbon dioxide, which is ready to be absorbed.

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