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

Kai-Lin Cai, Guo-Bing Wang, Li-Juan Xiong

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.

Carbon dioxide or nitrogen treatment

The CCL-228 cells were seeded onto 6-well plates (1x10⁶ 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 % CO₂ (CO₂ group) or in 95 % N₂ + 5 % CO₂ (N₂ 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 CO₂ for 15 min, with nitrogen for 90 min or 150 min, and then with CO₂ again for 15 min. After the gas treatment, all cells were incubated again in 5 % CO₂, 95 % air at 37 °C. The cultures were free of mycoplasma.

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 endogeneous peroxidase was blocked using 3 % hydrogen peroxyde for 10 min and the unspecified combined site was blocked with normal goat serum. Excessive blocking serum was drained and the samples were incubated at 40 °C 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 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 ILD±s. Results were then analyzed with Student’s *t*-test.

**RESULTS**

**Effects of CO<sub>2</sub> and N<sub>2</sub> on adhesive growth of CCL-228**

Sixty min of CO<sub>2</sub> treatment or 120 min of N<sub>2</sub> 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<sub>2</sub> and N<sub>2</sub> 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<sub>2</sub> or N<sub>2</sub> treatment

| Duration | Counts of suspension cells (>1000/µl) | Survival rate of suspension cells |
|----------|---------------------------------------|----------------------------------|
|          | CO<sub>2</sub> group | N<sub>2</sub> group | CO<sub>2</sub> group | N<sub>2</sub> group |
| 0 min    | 4.2±0.1                     | 0.88±0.01                     |
| 30 min   | 7.5±2.9                     | 4.9±2.5                       | 0.91±0.018                 | 0.85±0.022                 |
| 60 min   | 16.7±5.4<sup>a</sup>        | 5.2±0.7                       | 0.91±0.041                 | 0.89±0.021                 |
| 120 min  | 23.2±10.7<sup>b</sup>       | 8.5±1.4<sup>b</sup>           | 0.90±0.034                 | 0.91±0.023                 |
| 180 min  | 32.1±5.5<sup>c</sup>        | 7.7±3.3<sup>c</sup>           | 0.845±0.028                | 0.91±0.032                 |

<sup>a</sup>*P*<0.01, vs the 0 min in same group; <sup>b</sup>*P*<0.05, vs N<sub>2</sub> group at same time; <sup>c</sup>*P*<0.01, vs N<sub>2</sub> group at same time; *P*>0.05, vs 0 min in same group.

**Effects of CO<sub>2</sub> and N<sub>2</sub> on expression of E-cadherin in CCL-228 cell**

Both CO<sub>2</sub> and N<sub>2</sub> 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<sub>2</sub> treatment was significantly different from that after the same time periods of N<sub>2</sub> treatment. Comparing with the expression of E-cadherin before any gas treatment, 120 min or longer time of CO<sub>2</sub> treatment produced a very significant depression (*P*<0.01) and the same periods of N<sub>2</sub> treatment caused a significant depression (*P*<0.05).

**Effects of CO<sub>2</sub> and N<sub>2</sub> on expression of VEGF in CCL-228 cell**

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

**Effects of sequential treatment with CO<sub>2</sub> and N<sub>2</sub> 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).

**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[11,15]. Now many gastroenteric surgeries including cancer resection could be fulfilled under laparoscopy. 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[16], 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<sub>2</sub> or N<sub>2</sub> treatment. The rate of living cells in
significantly different from that by persistent carbon dioxide in the time between E-cadherin and VEGF expressions were the unabsorbable nitrogen. We found that, during the 120 min of CO₂ 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 CO₂ 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 the original lesions[11-15]. 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[26-28]. 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.

**REFERENCES**

1. Neuhaus SJ, Watson DJ, Ellis T, Rowland R, Rofe AM, Pike GK, Mathew G, Jamieson GG. Wound metastasis after laparoscopy with different insufflation gases. *Surgery 1998; 123:* 579-583
2. Schaeff B, Paolucci V, Thomopoulos J. Port site recurrences after laparoscopic surgery. *Dig Surg 1998; 15:* 124-134
3. Wexner S, Cohen SM. Port site metastases after laparoscopic colorectal surgery for cure of malignancy. *Br J Surg 1995; 82:* 295-298
4. Lacy AM, Delgado S, Garcia-Valdecasas JC, Castellis A, Pique JM, Grande L, Fuster J, Targarona EM, Pera M, Visa J. Port site metastases and recurrence after laparoscopic colectomy. *Surg Endosc 1998; 12:* 1039-1042
5. Mathew G, Watson DJ, Rofe AM, Baigrie CF, Ellis T, Jamieson GG. Wound metastases following laparoscopic and open surgery for abdominal cancer in a rat model. *Br J Surg 1996; 83:* 1087-1090
6. Gibson M, Byrd C, Pierce C, Wright F, Norwood W, Gibson T, Zibari GB. Laparoscopic colon resections: a five-year retrospective review. *Am Surg 2000; 66:* 245-248
7. Trokel MJ, Bessler M, Trear MR, Whelan RL, Nowygrud R. Preservation of immune response after laparoscopy. *Surg Endosc 1994; 8:* 1385-1387
8. Allendorf JD, Bessler M, Whelan RL, Trokel M, Laird DA, Terry MB, Trear MR. Postoperative immune function varies inversely with the degree of surgical trauma in a murine model. *Surg Endosc 1997; 11:* 427-430
9. Cho JM, LaPorta AJ, Clark JR, Schofield MJ, Hammond SL, Mallory PL. 2nd. Response of serum cytokines in patients undergoing laparoscopic cholecystectomy. *Surg Endosc 1994; 8:* 1385-1387
10. Wexner SD, Cohen SM, Johannsen OB, Nogueiras J, Jagelman DG. Laparoscopic colorectal surgery: a prospective assessment and current perspective. *Br J Surg 1993; 80:* 1602-1605
11. Evrard S, Falkenrodt A, Park A, Tassetti V, Mutter D, Marescaux J. Influence of CO₂ pneumoperitoneum on systemic and peritoneal cell-mediated immunity. *World J Surg 1997; 21:* 353-356
12. Murthy SM, Goldschmidt RA, Rao LN, Ammirati M, Buchmann T, Scanlon EF. The influence of surgical trauma on experimental metastasis. *Cancer 1989; 64:* 2035-2044
13. Da Costa ML, Redmond P, Boucher-Hayes DJ. The effect of laparotomy and laparoscopy on the establishment of spontaneous tumor metastases. *Surgery 1990; 124:* 516-525
14. Paik PS, Misawa T, Chiang M, Towson J, Im S, Ortega A, Beart RW Jr. Abdominal incision tumor implantation following pneumoperitoneum laparoscopic procedure vs. standard open incision in a syngeneic rat model. *Dis Colon Rectum 1998; 41:* 419-422
15. Ishida H, Murata N, Yamada H, Nakada H, Takeuchi I, Shimomura K, Fujioka M, Ieda Y. Pneumoperitoneum with carbon dioxide enhances liver metastases of cancer cells implanted into the portal vein in rabbits. *Surg Endosc 2000; 14:* 239-242
16. Dorudi S, Hanby AM, Poulsom R, Northover J, Hart IR. Level of expression of E-cadherin mRNA in colorectal cancer correlates with clinical outcome. *Br J Cancer 1995; 71:* 614-616
17. Jiang WG. E-cadherin and its associated protein catenins, cancer invasion and metastasis. *Br J Surg 1996; 83:* 437-446
18. Kinsella AR, Green B, Leptis GC, Hill CL, Bowie G, Taylor BA. The role of the cell–cell adhesion molecule E-cadherin in large bowel tumour cell invasion and metastasis. *Br J Cancer 1993; 67:* 904-909
19 Jiang WG. Cell adhesion molecules in the formation of liver metastasis. J Hepatobiliary Pancreat Surg 1998; 5: 375-382
20 Zhou YN, Xu CP, Han B, Li M, Qiao L, Fang DC, Yang JM. Expression of E-cadherin and beta-catenin in gastric carcinoma and its correlation with the clinicopathological features and patient survival. World J Gastroenterol 2002; 8: 987-993
21 Bruns CJ, Liu W, Davis DW, Shaheen RM, McConkey DJ, Wilson MR, Bucana CD, Hicklin DJ, Ellis LM. Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer 2000; 89: 488-499
22 Ellis LM, Takahashi Y, Liu W, Shaheen RM. Vascular endothelial growth factor in human colon cancer: biology and therapeutic implications. Oncologist 2000; 5(Suppl 1): 11-15
23 Kondo Y, Arii S, Mori A, Furutani M, Chiba T, Imamura M. Enhancement of angiogenesis, tumor growth, and metastasis by transfection of vascular endothelial growth factor into LoVo human colon cancer cell line. Clin Cancer Res 2000; 6: 622-630
24 Liu DH, Zhang XY, Fan DM, Huang YX, Zhang JS, Huang WQ, Zhang YQ, Huang QS, Ma WY, Chai YB, Jin M. Expression of vascular endothelial growth factor and its role in oncogenesis of human gastric carcinoma. World J Gastroenterol 2001; 7: 500-505
25 Tao HQ, Lin YZ, Wang RN. Significance of vascular endothelial growth factor messenger RNA expression in gastric cancer. World J Gastroenterol 1998; 4: 10-13
26 Jacobi CA, Sabat R, Bohm B, Zieren HU, Volk HD, Müller JM. Pneumoperitoneum with carbon dioxide stimulates growth of malignant colonic cells. Surgery 1997; 121: 72-78
27 Jacobi CA, Wenger F, Sabat R, Volk T, Ordemann J, Muller JM. The impact of laparoscopy with carbon dioxide versus helium on immunologic function and tumor growth in a rat model. Dig Surg 1998; 15: 110-116

Edited by MajY