Changes in CO_2 Concentration Increase the Invasive Ability of Colon Cancer Cells

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Abstract. *Background/Aim: We studied the effects of CO_2 concentration changes on the invasive ability of colon cancer cells. Materials and Methods: Colon cancer cell lines and human samples derived from a peritoneal metastasis were incubated in a hypercapnic environment, followed by incubation in 5% CO_2. The invasive ability of colon cancer cells incubated with CO_2 were analyzed using an invasion assay system. Results: In comparison with the colon cancer cell lines incubated in 5% CO_2 only, the invasive ability of cells increased in all the colon cancer cell lines subjected to incubation in 20% CO_2 followed by incubation in 5% CO_2, with a concomitant increase in the mRNA expressions of matrix metalloproteinase-2 (MMP2) and MMP9. The invasive capability of peritoneal metastatic cells in the human-derived specimen also increased on CO_2 concentration changes. Conclusion: CO_2 concentration changes enhanced the invasive capacity of colon cancer cells.*

Laparoscopic surgery has recently become one of the most widespread operational procedures in Japan (1). In 1990, a cholecystectomy was the first laparoscopic surgery performed in Japan, and thereafter, various organs have been operated on, such as the large intestine, lung, uterus, and ovary (1). At present, laparoscopic surgery is being used not only for benign diseases but also for malignant tumours. According to the 2010 report of the Japanese Society for Endoscopic Surgery, approximately 120,000 cases were treated by laparoscopic surgery across Japan, and approximately 20,000 of these cases were those of colorectal cancer (2). The Japanese Clinical Practice Guidelines for Colon Cancer recommend laparoscopic surgery for colon cancer up to stage II; however, the application of laparoscopic surgery has been extended to more advanced cancers, partly because of improvement in surgical skills (1, 2). In general, CO_2 is used to expand the abdominal cavity to improve the visual field for surgery, leading to CO_2-related adverse effects such as tumour recurrence or exacerbation of progression. Among the various reports available, some studies have reported adverse effects of CO_2, such as inhibition of macrophage activity in the peritoneum and the likelihood of tumour growth because of CO_2 introduction into the peritoneum (3-5), while other studies have mentioned that incubation with high CO_2 concentration-alone has no effect on colorectal cancer cells (6). In most laparoscopic surgeries for colon cancer, the primary lesion is detached or cut-off in the abdominal cavity insufflated with CO_2, and then a small incision is made to remove the target organ from the abdominal cavity, followed by anastomosis. In this situation, a small abdominal incision results in exposure to a high CO_2 concentration. Accordingly, we examined the effect of changes in CO_2 concentration on the invasive ability of cancer cells *in vitro* and obtained interesting results that are discussed below.

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Materials and Methods

Cell culture. The human colon cancer cell lines SW620, Colo205, and HCT116 were cultured at 37˚C in 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (7).

CO_2 treatment. Cells were seeded (5×10^5) into 6-cm dishes in triplicate. The following day, cancer cells were incubated with 5% CO_2-alone, with 20% CO_2-alone for six hours, or with 20% CO_2 for six hours followed by 5% CO_2 incubation for six hours

Tumor cell invasion assay. The invasion of colon cancer cells was analyzed by a Biocoat Matrigel 6-well invasion chamber (BD Biosciences, San Jose CA, USA) (8). In the upper insert, 2.5×10^4 cells/well in RPMI-1640 medium without fetal bovine serum were plated, and complete medium was added to the lower insert, both then were cultured for 48 h. After incubation, the colon cancer cells remaining in the upper insert were removed using a swab and the colon cancer cells which had infiltrated into the lower insert were fixed and stained with the Diff-Quik stain (Sysmex, Kobe Japan). The experiments were repeated three times.

Table I. *Characteristics of colorectal cancer with peritoneal metastasis.*

Case	Years	M/F	Primary location	Histological type*	Metachronous or synchronous
1	50	F	Rs	Tub2	Metachronous
2	56	M	S	Poor	Synchronous
3	75	M	RbP	Poor	Synchronous
4	35	F	S	Tub2	Synchronous
5	68	M	A	Poor	Synchronous

*According to reference 26. Rs: Rectosigmoid; S: sigmoid colon; RbP: rectum (below the peritoneal reflection) and proctos; A: ascending colon.

RNA extraction and Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from colon cancer cells using ISOGEN (Wako, Tokyo Japan). Single-strand cDNA prepared from 3 μg of total RNA using Prime Script RT reagent kit (Takara, Otsu Japan) was used as the template for the PCR (9). The primers for PCR to amplify matrix metalloproteinase-2 (*MMP2*) gene-coding regions were as follows: 5' primer, *MMP2*-AX: ACCCATTTAC ACCTACACCAAG; 3' primer, *MMP2*-BX: GTATACCGCATCAA TCTTTTCCG. The primers for PCR to amplify MMP9 gene-coding regions were as follows: 5' primer, *MMP9*–AX: TGGGC ACGTGACCTATGACAT; 3' primer, *MMP9*–BX: GCCCAGCCCAC CTCCACTCCTC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as internal PCR control with 5'-GGGGAGCCAAAAGGGTCATCA TCT-3' as the sense primer and 5'-GACGCCTGCTTCACCACCTT CTTG-3' as the antisense primer. Thirty cycles of denaturation (94˚C, 1 min), annealing (50˚C, 1.5 min), and extension (72˚C, 2 min) were carried out in a thermal cycler (PTC-100, Programmable Thermal Controller; MJ Research Inc., Watertown MA, USA). Ten microlitres of the PCR product were resolved by electrophoresis in 1.2% agarose gel. The sequencing was performed on PCR products that revealed the bands in RT-PCR analysis. Ethidium bromide staining of the gels identified a band of *MMP2* and -9 mRNA. To ensure reproducibility, all PCR amplifications were performed in triplicate

Peritoneal metastatic cells of human colon cancer. A total of 215 patients with colon cancer who had undergone colectomy at the First department of Surgery, University of Fukui (Japan) between January 2011 and June 2012 were included in this study. Five patients suffered from peritoneal metastasis, histologically-confirmed by peritoneal cytology (Table I).

Cancer cells in ascitic fluid from patients were washed with phosphate-buffered saline (PBS), and the resulting cancer cell suspension was diluted to 1.0×10^6 cells/ml. The cells were analyzed by using a Biocoat Matrigel 6-well invasion chamber.

Statistical considerations. Characteristics of the three treatment arms were compared using the Paired *t*-test. Values of <0.05 were considered statistically significant.

Results

Changes in the invasive ability of colon cancer cell lines induced by high CO_2 concentration. Alterations in the invasive ability of colon cancer cell lines were assessed using an invasion assay system. Colon cancer cell lines incubated with 5% CO_2 were used as control. Under hypercapnic conditions (20% CO_2, six hours), SW620, Colo205, and HCT116 cell line invasion increased by 20-110% (SW620: 6.8 *vs*. 5.7 cells for the control, Colo205: 3.6 *vs*. 1.7 cells for the control, HCT116: 12.4 *vs*. 8.0 cells for the control). However, these differences did not reach statistical significance (Figures 1 and 2). The cell invasive ability significantly increased when cells were incubated with 20% CO_2, as compared with 5% CO_2.

Biphasic alteration of CO_2 concentration-induced changes in the invasive ability of colon cancer cell lines. The cell invasive ability of all lines increased significantly after additional incubation with 5% CO_2 following that with 20% CO_2, compared with incubation with 5% CO_2 alone (Figure 1 and 2). In SW620, Colo205, and HCT116 cells incubated in hypercapnic and then a normal CO_2 environment, invasion was 2.5- to 7.9-fold that of cells incubated only in the normal CO_2 environment (SW620: 14.2 *vs*. 5.7 cells for the control, Colo205: 13.4 *vs*. 1.7 cells for the control, HCT116: 36.1 *vs*. 8.0 cells for the control). Cell invasive ability significantly increased on additional incubation with 5% CO_2, following incubation with 20% CO_2, as compared with incubation with 5% CO_2 alone.

Increased mRNA expressions of MMP2 and MMP9 in colon cancer cell lines after biphasic CO_2 incubation. In all three colon cancer cell lines, the mRNA expressions of *MMP2* and *MMP9* increased when cells were incubated first with 20% CO_2 and then with 5% CO_2, compared to the expression after incubation with 5% CO_2 alone (Figure 3).

Invasive ability of peritoneal metastastic cells of human colon cancer after biphasic CO_2 incubation. Figures 4 and 5 show the invasive ability of peritoneal metastatic cells of human colon cancer when incubated with 5% CO_2, only, with 20% CO_2 for six hours only, and with 20% CO_2 for six hours followed by incubation with 5% CO_2 for six hours. Under hypercapnic conditions (20% CO_2, six hours), cell invasion increased by 25-108%. The number of cells which had invaded under both concentrations of CO_2 increased by 1.75-6.4-fold when compared to incubation with 5% CO2 alone.

Discussion

CO_2 is frequently used to extend the visual field for laparoscopic surgery, but there are only a few detailed reports on the effects of CO_2 on colorectal cancer cells. At present, the applications of laparoscopic surgery are expanding, particularly in Japan. There are also reports on its application to treat advanced cancer. According to the literature, peritoneal lavage cytology revealed the existence of cancer

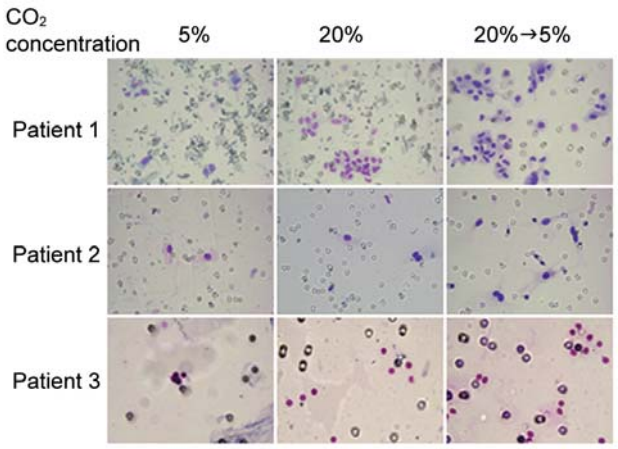

Figure 1. *Photograph of invading colon cancer cells. Using the Matrigel assay, the invasive colon cancer cells in the lower chamber were stained and identified by microscopy.*

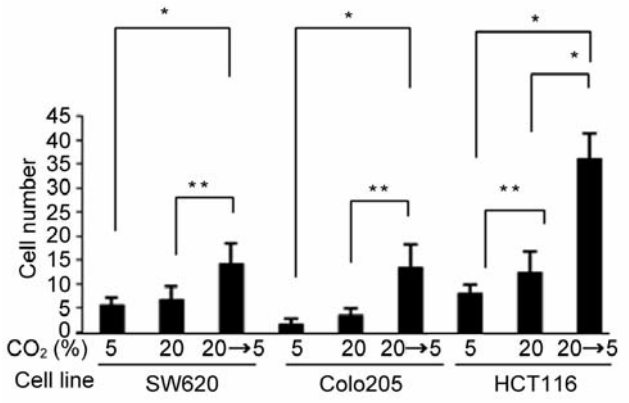

Figure 2. *The number of invading colon cancer cells. The number of invaded colon cancer cells was quantified using Matrigel assay. Colon cancer cells invasive ability significantly increased additional incubation with 5% CO_2 after that with 20% CO_2 as compared with that with 5% CO_2-alone. Results are presented as the mean±SD (n=4). *p<0.01. **p<0.05.*

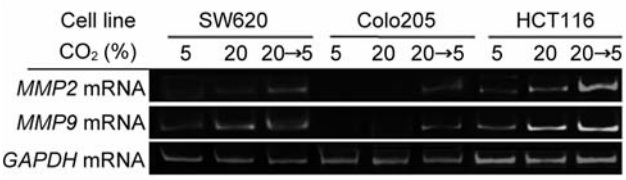

Figure 3. *Matrix metalloproteinase (MMP) mRNA expression in colon cancer cells. The expression of MMP mRNA was detected by reverse transcription-polymerase chain reaction. The mRNA expression of MMP2 and MMP9 increased when cells were incubated first with 20% CO_2 and then additional with 5% CO_2, as compared to the mRNA expression after that with 5% CO_2-alone.*

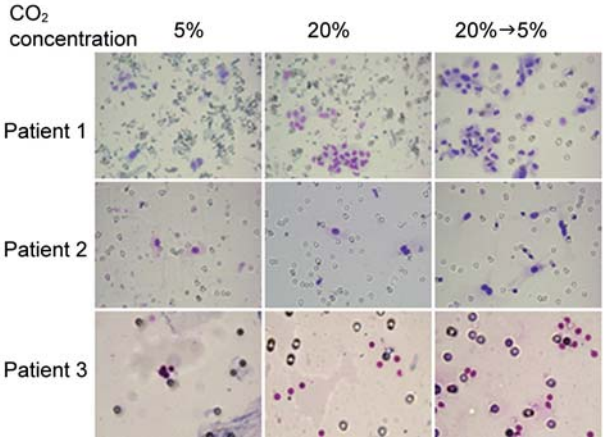

Figure 4. *Photograph of invading peritoneal metastatic cells of human colon cancer. Using the Matrigel assay, the invasive peritoneal metastatic cells in the lower chamber were stained and identified by microscopy.*

cells in 20-40% of stage I-III colorectal cancer cases (10-12). Furthermore, an increased invasive capacity of cancer cells elevates the risk of recurrence/metastasis of colorectal cancer and other malignant tumour types (13-17). Detailed reports on the effects of changes in CO_2 concentrations on the invasive ability of colorectal cancer cells are, however, lacking. The effects of high CO_2 may be time-dependent and may only be uncovered following a return to normal CO_2 concentrations. Accordingly, we considered it necessary to make further investigations. We found that the cellular invasive ability of colon cancer cell lines was strengthened by changing the incubation conditions from highly concentrated CO_2 to a normal concentration. Furthermore, we determined that such a protocol enhanced the gene expression profile of the matrix metalloproteinase family members (18-20), MMP2 and MMP9, which are considered to be critical factors in cancer invasion (21-24). We hypothesize that these stress-induced changes in gene expression are only revealed once the stressful stimuli are removed (25), and this might explain why significant differences in invasive potential were only observed in the experiments involving high and low CO_2 concentrations. Similar results were found for the peritoneal cells originating from colon cancer: changes in CO_2 concentration seemed to greatly-affect the invasive ability of cancer cells.

When laparoscopic colectomy is suggested for advanced cancer cases, the presence of peritoneal cancer cells and abdominal CO_2 concentrations may have to be considered in determining the operative procedure.

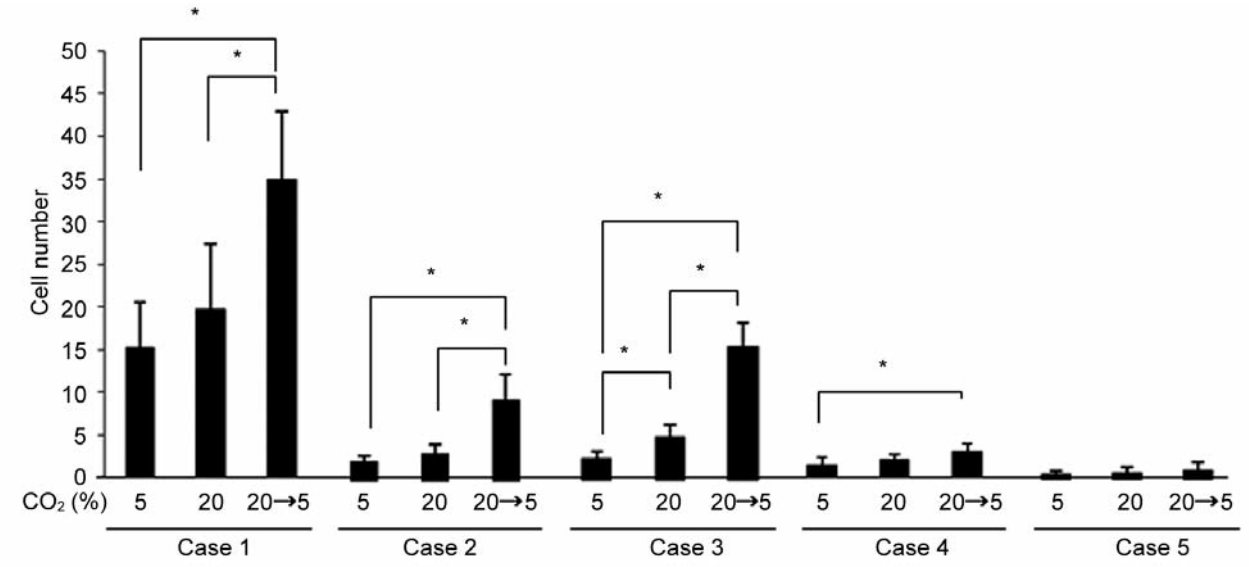

Figure 5. *The number of invaded peritoneal metastatic cells of human colon cancer. The number of invaded peritoneal metastatic cells was quantified using the Matrigel assay. The number of invading cancer cells when incubated in both 20% CO_2 and 5% CO_2 increased 1.75- to 6.4-fold when compared to the number of invading by cancer cells in 5% or 20% CO_2 alone. Results are presented as the mean±SD (n=5). *p<0.05.*

Conflict of Interests

The Authors do not have any significant financial interest in any company making any products discussed in the article. The Authors report no conflicts of interest.

Authors' declaration

All the Authors have read the manuscript and have approved this submission.

We attest that the research was performed in accordance with the humane and ethical rules for human experimentation that are stated in the Declaration of Helsinki.

The article is original, is not under consideration by any other journal and has not been previously published.

Refereces

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