Impact of Laparoscopic and Conventional Surgery on Kupffer Cells, Tumor-Associated CD44 Expression, and Intrahepatic Tumor Spread

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Background: The oncologic feasibility of laparoscopic surgery for the cure of colorectal cancer is under debate. The effect of laparoscopic colorectal cancer resection on hepatic tumor spread has not yet been clarified.

Hypothesis: Laparoscopic surgery affects cell-mediated immune response and hepatic tumor spread dependent on intraperitoneal insufflation.

Methods: Thirty WAG/Rij rats were randomized into 3 operative groups: carbon dioxide (CO2) laparoscopy (n=10), “gasless” laparoscopy (n=10), and laparotomy (n=10). To induce liver metastases, 50,000 CC531 colon carcinoma cells were injected into the portal vein during either laparoscopy or laparotomy. Twenty-eight days after injection, specimens were explanted, sectioned, and examined immunohistochemically for CC531 tumor cells (monoclonal antibody CC52), CD44v5, v6 (monoclonal antibody OX49), and Kupffer cells (monoclonal antibody HIS36). For quantification, a morphometric analysis system was applied. Data were analyzed using the Kruskal-Wallis, Dunn, and Holm tests.

Results: No statistically significant differences in hepatic tumor growth were found between CO2 laparoscopy and laparotomy (P=.37). However, compared with CO2 laparoscopy and laparotomy, a significant decrease in intrahepatic tumor growth was found after gasless laparoscopy (P=.02). Kupffer cells had significantly decreased after CO2 laparoscopy and laparotomy compared with after gasless laparoscopy (P<.001 and P=.002, respectively). CD44v5, v6 expression was significantly increased after CO2 laparoscopy and laparotomy compared with after gasless laparoscopy (P=.002 and P=.05, respectively).

Conclusions: Hepatic resistance to tumor growth is best preserved by gasless laparoscopy as opposed to CO2 laparoscopy or laparotomy. The amount of intra-abdominal pressure with circulatory changes rather than the used gas may explain this finding. On the other hand, conventional laparoscopy vs laparotomy did not preserve hepatic immune function.

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IN PATIENTS with colorectal cancer, the appearance of liver metastasis represents a major clinical problem, decreasing life expectancy substantially.1 Metastatic spread to the liver is caused by circulating tumor cells in the portal system, which were separated from the primary focus. After spreading from the portal system, malignant cells must pass through each step of the metastatic process, that is, they must attach to, and extravasate through, the capillary wall; adjust to the new environment; and proliferate.2 Most tumor cells cannot successfully pass through the stepwise metastatic process and will be cleared by various host defense mechanisms. Thus, only a few of the circulating tumor cells survive. The mononuclear phagocyte system (MPS), which is located more than 90% in the liver, is involved in this physiologic defense mechanism against bacterial infection and tumor spreads.3-5 Surgical intervention can reduce the activity of the MPS and compromise endogenous resistance to malignant cell growth and, therefore, may facilitate metastatic spread.6,7

Recent experimental studies6 have shown that phagocyte activity of the MPS expressed as antigen elimination by the liver is reduced during laparoscopic surgery using a carbon dioxide (CO2) pneumoperitoneum compared with “gasless” laparoscopy. Pneumoperitoneum and elevated intra-abdominal pressure compromise the splanchnic blood flow and the afferent circulation of the liver.8-11 However, whether elevated intra-abdominal pressure during CO2 laparoscopy is responsible for alterations of local defense mechanisms in the liver has not yet been clarified. Besides hemodynamic changes and hepatic impairment due to increased intra-abdominal pressure, CO2 insufflation is suspected of directly promoting tumor cell proliferation.12,13 Alternative insufflation gases might reduce malignant cell growth.13

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The aims of the present study are to examine the effect of different surgical techniques on hepatic tumor spread in a standardized rat model for colorectal liver metastases\textsuperscript{14,15} and to analyze whether CO\textsubscript{2} insufflation contributes to hepatic alterations. Colon cancer cells were injected into the portal vein of rats during laparotomy, gasless laparoscopy, and laparoscopy using a pneumoperitoneum with CO\textsubscript{2}. Liver specimens were examined immunohistochemically for tumor cell growth, Kupffer cells (KCs), and the presence of tumor-associated molecules.

**METHODS**

**ANIMALS**

Thirty male WAG/Rij rats (Charles River GmbH, Sulzfeld, Germany) weighing 150 to 180 g were used. The animals were accustomed to an environment with a controlled climate and light cycle, and they had free access to standard laboratory rat chow and tap water ad libitum. All experiments were performed according to the regulations of the National Research Council and following the guidelines for care and use of laboratory animals.

**TUMOR**

The CC531 cell line is a 1,2-dimethylhydrazine–induced, weakly immunogenic, moderately differentiated rat colon adenocarcinoma, transplantable in syngeneic WAG/Rij rats. The cells were cultured in plastic culture flasks (Falcon Primaria; Becton Dickinson, Franklin Lakes, NJ) in RPMI 1640 medium (Gibco BRL, Karlsruhe, Germany) supplemented with 2\% HEPES buffer solution, 1\% streptomycin and penicillin, and 5\% fetal calf serum (Gibco BRL) at 37°C in a 5\% CO\textsubscript{2} atmosphere. After 62 passages, in vitro cells were washed with phosphate-buffered saline solution (Gibco BRL) at 37°C and centrifuged for 5 minutes at 1200 rpm. After resuspension in RPMI 1640 medium, the number and viability of tumor cells were measured using the trypan blue method. Viability always exceeded 95\%. The cell suspension was diluted in RPMI 1640 medium to obtain a concentration of 10000 cells/mL.

**OPERATIVE GROUPS AND ANESTHESIA**

Thirty rats were randomized into 3 operative groups: CO\textsubscript{2} laparoscopy (n=10), gasless laparoscopy (n=10), and open laparotomy (n=10). Anesthesia was performed by intraperitoneal injection of a ketamine hydrochloride (50 mg/kg) mixture. Liver peritoneum with CO\textsubscript{2}. Liver specimens were examined immunohistochemically for tumor cell growth, Kupffer cells (KCs), and the presence of tumor-associated molecules.

**LAPAROSCOPIC TUMOR CELL INOCULATION**

Laparoscopy was performed either by installing a CO\textsubscript{2} pneumoperitoneum with a constant intra-abdominal pressure of 7 mm Hg using an electronic insufflator (Storz, Tuttlingen, Germany) or by abdominal wall lift using fixable retraction wires.\textsuperscript{16} An arthroscopic sheath with an insufflation side port (Aesculap) was inserted to prepare and expose the portal vein. Forty-five minutes after setup, a 0.4 × 4.0-mm cannula (Sterican; B. Braun, Melsungen, Germany) was introduced percutaneously into the abdomen to puncture the portal vein. The vessel was carefully fixed, and tumor cell suspension was injected under laparoscopic guidance. To prevent bleeding, the vessel was compressed for 90 seconds using a cotton swab. Laparoscopy was continued for another 45 minutes, for a total operating time of 90 minutes.

**OPEN TUMOR CELL INOCULATION**

A 6-cm midline laparotomy was performed. The portal vein was prepared and exposed. Forty-five minutes later, tumor cell suspension was injected using the same 0.4-mm cannula. To prevent bleeding, the puncture site was compressed for 90 seconds. After another 45 minutes, the abdomen was closed in 2 layers using a running suture.

**ANALYSIS**

The animals were humanely killed 28 days after surgery. For immunohistochemical and microscopic analyses, the liver was perfused in situ for 5 minutes with 5 mL of HBSS solution (Gibco BRL). After explantation, the median and right liver lobes were snap frozen in isopentane and stored at \textdegree C.

**MONOCLONAL ANTIBODIES**

Primary monoclonal antibody (mAb) CC52 reacting with CC531 colon carcinoma cells was used. Primary mAb HIS36 reacting with ED2-like antigen (Pharmingen, San Diego, Calif), which is found on tissue macrophages but not on monocytes, was used to stain KCs. Primary mAb OX49 reacting with glycoprotein CD44 expressed on leukocytes, T-cell blasts, and B-cell blasts was obtained from Pharmingen. Secondary rabbit–anti-mouse peroxidase-conjugated antibodies reacting with mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM were obtained from DAKO, Glostrup, Denmark. Secondary swine–anti-rabbit peroxidase-conjugated antibodies reacting with rabbit immunoglobulins were also obtained from DAKO.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Liver cryostat sections of 4 µm were air dried for 24 hours and fixed in acetone for 10 minutes at room temperature. Standardized immunohistochemical analysis was performed using a staining machine (Midas E MMS; Diagnostic Systems Inc, Gibbstown, NY).

Cryostat sections were incubated for 1 hour at 37°C with primary mAb CC52 (1:400), mAb HIS36 (1:700), or mAb OX49 (1:600), which has been examined in pilot tests. After rinsing with water for 3 minutes and with phosphate-buffered saline solution for 2 minutes, sections were incubated for 15 minutes with secondary rabbit–anti-mouse peroxidase-conjugated antibodies (1:150). After rinsing with water for 3 minutes and with phosphate-buffered saline solution for 2 minutes, sections were incubated for 15 minutes with swine–anti-rabbit peroxidase-conjugated antibodies (1:75). After rinsing with water for 3 minutes and with phosphate-buffered saline solution for 2 minutes, sections were developed and developed in 3-amino-9-ethylcarbazole.

Cryostat sections were developed in 3-amino-9-ethylcarbazole solution (dimethylformamide, sodium acetate [pH 5.0], and 0.01% peroxidase) for 15 minutes. After rinsing with water for 10 minutes, sections were counterstained for 2 minutes with hematoxylin and mounted in glycerin gelatin.

**MICROSCOPIC EVALUATION**

Liver cryostat sections were analyzed microscopically using a morphometric analysis system (CBA 8000-Manager; Leica, Gibbstown, NJ).
Intrahepatic tumor growth, Kcs, and tumor-associated expression of CD44v5, v6 expression after carbon dioxide laparoscopy, gasless laparoscopy, and laparotomy*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Carbon Dioxide Laparoscopy (n = 10)</th>
<th>Gasless Laparoscopy (n = 10)</th>
<th>Laparotomy (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC531 (tumor), %</td>
<td>5.05 ± 1.12</td>
<td>1.06 ± 1.92†</td>
<td>5.41 ± 0.22</td>
</tr>
<tr>
<td>Kupffer cells, %</td>
<td>0.70 ± 0.57</td>
<td>2.71 ± 0.57†</td>
<td>1.23 ± 0.14</td>
</tr>
<tr>
<td>CD44v5, v6, %</td>
<td>0.72 ± 0.19</td>
<td>0.18 ± 0.10†</td>
<td>0.43 ± 0.28</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SD. Statistical analysis was performed using the Kruskal-Wallis, Dunn, and Holm tests.
†P < .05 vs carbon dioxide laparoscopy and vs laparotomy.

STATISTICAL ANALYSIS

Differences among the 3 groups were analyzed using the non-parametric tests of Kruskal-Wallis, Dunn, and Holm. Statistical significance was set at P < .05.

RESULTS

Intrahepatic tumor cell growth, Kcs, and tumor-associated expression of CD44v5, v6 molecules were investigated by immunohistochemical analysis 28 days after intraportal tumor cell injection in rats. Intrahepatic growth of CC531 colon adenocarcinoma cells was found in all groups. Although CO2 laparoscopy did not result in reduced tumor cell growth compared with laparotomy, gasless laparoscopy was related to reduced tumor cell growth. Morphometric analysis showed a significantly lower degree of staining of CC531 tumor cells after gasless laparoscopy than after CO2 laparoscopy (P = .007) or laparotomy (P < .001). No significant differences in CC531 staining and hepatic tumor growth were found when comparing CO2 laparoscopy and laparotomy (P = .47) (Table, Figure 1, and Figure 2).

Kupffer cells were better preserved after gasless laparoscopy than after CO2 laparoscopy or laparotomy. A significantly higher degree of staining of hepatic tissue macrophages was found after gasless laparoscopy than after CO2 laparoscopy (P < .001) or laparotomy (P = .002). No significant differences in KC counts were found between CO2 laparoscopy and laparotomy (P = .55) (Table and Figure 3).

Expression of tumor-associated glycoproteins was found in all tumor nodes but not in normal liver tissue. Morphometric analysis showed that expression of CD44v5, v6 was significantly reduced after gasless laparoscopy compared with after CO2 laparoscopy (P = .002) or laparotomy (P = .05). No significant differences were found between CO2 laparoscopy and laparotomy (P = .2) (Table and Figure 4).

COMMENT

The present study investigated for the first time the effect of different laparoscopic and open surgical procedures on hepatic immune function, expression of tumor-associated molecules, and metastatic tumor take in the liver.

Although the laparoscopic approach to colorectal surgery has been shown to be technically feasible, the oncologic feasibility of CO2 laparoscopy for cure of malignancies is still under debate. First, concerns about the
In the field of colorectal cancer, metastatic disease to the liver represents a major clinical problem, with a poorer prognosis for the patients. The liver is the first site for metastatic tumor spread. Incidences of colorectal liver metastases range from 35% to 50%, and it has been shown that liver metastases are present in 25% of patients at the time of the initial colorectal resection. Furthermore, in a recent study it was possible to demonstrate perioperatively circulating tumor cells in patients with colorectal cancer. The formation of such hematogenous metastases derives from loss of cell-cell adhesion at the primary site, adhesion to the basement membrane, invasion of the basement membrane and extracellular matrix, and intravasation. Once bloodborne tumor cells circulate to distant organs, such as the liver, they adhere to the endothelium, extravasate, and, finally, establish secondary foci with neovascular formation.

In the process of cell detachment and cell attachment during the metastatic cascade, a varying number of cell adhesion molecules are involved. It is considered that the cell-cell adhesion molecule CD44, a glycoprotein with several physiologic cellular functions, is implicated in tumor progression, invasiveness, and metastatic potential of malignant neoplasms. Substantial up-regulation of CD44v5, v6 expression was found in colorectal cancer compared with normal epithelium. However, even after having successfully passed through the stepwise metastatic process, only a few colorectal cancer cells will give rise to solid liver metastases, largely owing to hepatic immunocompetent cells, mainly consisting of tissue macrophages (KCs). Kupffer cells can kill tumor cells and are present in the sinusoids or proliferate in the liver parenchyma and play an important tumoricidal role. Therefore, preservation of the hepatic immune function at the time of surgical tumor manipulation is important for the defense mechanisms against metastatic intrahepatic tumor spread.

Results of recent studies have indicated that the MPS, which is located more than 90% in the liver, is impaired during CO2 laparoscopy. The potential benefit conferred by minimal-access surgery on the postoperative immune response seems to be negated by installation of a CO2 pneumoperitoneum when investigating hepatic immune function. If malignant cells are present in the portal system at the critical time of colorectal resection, reduced MPS activity might facilitate metastatic tumor spread. The mechanisms of CO2 laparoscopy leading to impairment of KCs and hepatic immune function have not been investigated to date, to our knowledge.

Installation of a CO2 pneumoperitoneum during laparoscopic surgery has been shown to affect splanchnic microcirculation and microcirculation. Eleftheriadis et al demonstrated that a significant alteration of hepatic microcirculation takes place during laparoscopic standard procedures in humans. Jakimovicz et al described a portal venous blood flow reduction of 53% during laparoscopic cholecystectomy in humans. Similar experimental data were found in a study by Gutt and Schmandra. The installation of a CO2 pneumoperitoneum showed a linear decrease of portal venous blood flow with elevating intra-abdominal pressure.

Recent experimental studies investigated the growth of colorectal liver metastases after laparoscopic surgery using a CO2 pneumoperitoneum. Ishida et al demonstrated that CO2 laparoscopy significantly increases hepatic metastases compared with open surgery. In contrast, data from Gutt et al suggest that gasless laparoscopy was associated with reduced hepatic tumor spread compared with CO2 laparoscopy or laparotomy. The reasons for these findings have not yet been clarified.

In the present study, laparotomy, gasless laparoscopy, and laparoscopy with CO2 pneumoperitoneum were performed without any complications in 30 WAG/Rij rats to investigate KCs, CD44 expression, and hepatic tumor spread by immunohistochemical analysis. The analysis of KCs showed that CO2 laparoscopy is associated not only with systemic immunologic benefits but also with negative effects on hepatic immune functions. Kupffer cells were not better preserved after laparoscopy with CO2 pneumoperitoneum than after laparotomy. Only gasless laparoscopy was associated with better preservation of KCs. Expression of CD44 was found to be related to intrahepatic tumor growth. No significant differences were discerned between CO2 laparoscopy and laparotomy. Only after gasless laparoscopy was CD44 expression reduced. Intrahepatic tumor growth was reduced only after gasless laparoscopy, whereas no differences were found between laparotomy and laparoscopy with CO2 pneumoperitoneum.

In conclusion, our data demonstrate that circulatory changes due to the amount of intra-abdominal pressure rather than the used gas during conventional laparoscopy play an important role on hepatic tumor take and

Figure 4. Tumor-associated cell adhesion molecule CD44v5, v6 after gasless laparoscopy. CD44v5, v6 were stained immunohistochemically with primary monoclonal antibody OX49.
The present study investigates the effect of laparoscopic and conventional surgery on the development of colorectal liver metastases. On the background of wound metastases, local recurrences, intra-abdominal tumor cell dissemination, and a controversial debate about the oncologic safety of minimally invasive cancer surgery, a standardized experimental model was used to simulate hematogenous tumor spread under laparoscopic conditions.

The liver is the first site for metastatic spread of colorectal carcinomas. Metastatic disease of the liver generally determines the outcome of patients with colorectal cancer. Therefore, the development of liver metastases might represent a valid indicator for oncologic advantages of different surgical techniques. However, the effect of laparoscopic vs open surgery on this issue has not been investigated, and sufficient data from randomized controlled clinical trials are not available, to our knowledge.

The impact of laparoscopic insufflation with carbon dioxide, “gasless” laparoscopy, and open surgery on the development of implanted colorectal liver metastases was investigated in the rat. Hepatic tumor cell growth was analyzed macroscopically and microscopically. Furthermore, tumor-associated molecules and Kupffer cells were investigated using immunohistochemical analysis.

The results of the present study should provide clinicians with the current data of basic science on the oncologic consequences of laparoscopic surgery and possible mechanisms for hematogenous metastatic tumor spread.

The results of the present study demonstrate for the first time that circulatory changes due to increasing intra-abdominal pressure increase hepatic tumor spread and associated CD44 expression. The benefits of minimal-access surgery on systemic immune function seem to be compromised by local effects of gas insufflation. Our findings suggest that reticuloendothelial depression following surgery.

REFERENCES


Statement of Clinical Relevance

the development of liver metastasis. Furthermore, gasless laparoscopy compared with conventional laparoscopy or laparotomy best preserved hepatic immune function. However, further studies are required to characterize the effect and mechanism of different intra-abdominal pressures with different insufflation gases on intrahepatic tumor take, local immune response, and expression of tumor-associated molecules and growth factors.

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