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, 50000 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. 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.

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. 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.6,11 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
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 and to analyze whether CO₂ 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 CO₂. 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). After 62 passages, in vitro cells were washed with phosphate-buffered saline solution (Gibco BRL), trypsinized for 5 minutes 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 dye exclusion method. Viability always exceeded 95%. The cell suspension was diluted in RPMI 1640 medium to obtain a concentration of 10,000 cells/mL.

**OPERATIVE GROUPS AND ANESTHESIA**

Thirty rats were randomized into 3 operative groups: CO₂ 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) and xylazine hydrochloride (5 mg/kg) mixture.

**LAPAROSCOPIC TUMOR CELL INOCULATION**

Laparoscopy was performed either by installing a CO₂ pneumoperitoneum with a constant intra-abdominal pressure of 7 mm Hg using an electronic insufflator (Storz, Tuttingen, Germany) or by abdominal wall lift using fixable retraction wires. An arthroscopic sheath with an insufflation side port (Aesculap, Tuttingen), including a 5-mm arthroscope (Aesculap), and two 3-mm synthetic ports were implanted. Three-millimeter endoscopic instruments (Aesculap) were 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 −80°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 using a staining machine (Midas E MMS; Diagnostic Systems Inc, Gibbstown, NY).

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**MICROSCOPIC EVALUATION**

Liver cryostat sections were analyzed microscopically using a morphometric analysis system (CBA 8000-Manager; Leica, Germany). Liver 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 using a staining machine (Midas E MMS; Diagnostic Systems Inc, Gibbstown, NY).
Wetzlar, Germany). The intensity of the stained antibody was measured using a computer in 20 microscopic fields of each cryostat section. Macrophages, CC531 tumor cells, and CD44v5, v6 adhesion molecules were evaluated.

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 adhesion 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 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 ($P<.001$) 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 conclusion, our data demonstrate that circulating 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
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 hepatic resistance against tumor growth is better preserved by gasless laparoscopy than by either conventional laparoscopy or laparotomy.

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