Comparative Analysis of Tumor Cell Dissemination in Mesenteric, Central, and Peripheral Venous Blood in Patients With Colorectal Cancer

Moritz Koch, MD; Jürgen Weitz, MD; Peter Kienle, MD; Arel Benner, MSc; Frank Willeke, MD; Thomas Lehner, MD; Christian Herfarth, MD; Magnus von Knebel Doeberitz, MD

Background: Metastatic disease in colorectal cancer results from hematogenic dissemination of tumor cells. This dissemination can be explained by 2 concepts: (1) regional spread of tumor cells via portal venous drainage into the liver as the first site of metastasis and (2) early spread of tumor cells into central and peripheral venous blood as evidence of systemic hematogenic tumor cell dissemination.

Hypothesis: Tumor cell detection in different blood compartments could help to understand the predominant pattern of hematogenic tumor cell dissemination in colorectal cancer.

Design: Prospective consecutive series.

Setting: University hospital.

Patients and Methods: Mesenteric, central, and peripheral venous blood samples from 40 patients with colorectal cancer were examined by cytokeratin 20 reverse transcriptase polymerase chain reaction.

Main Outcome Measures: Sensitivity and specificity of cytokeratin 20 reverse transcriptase polymerase chain reaction, and frequency of tumor cell detection in different blood compartments.

Results: Tumor cells were found in mesenteric venous blood of 20 of 40 patients, central venous blood of 6 of 40 patients, and peripheral venous blood of 2 of 19 patients. The detection rate in mesenteric venous blood was significantly higher than that in central and peripheral venous blood (P<.001).

Conclusions: The significantly higher detection rate in mesenteric venous blood emphasizes the importance of the filter function of the liver for circulating tumor cells in portal venous blood. Tumor cell detection in central and peripheral venous blood, however, shows that this filtering process is limited and indicates early systemic hematogenic tumor cell dissemination in colorectal cancer.

PATIENTS AND METHODS

BLOOD SAMPLES

Immediately after laparotomy and before tumor mobilization, 3 blood samples (each 10 mL in EDTA) were drawn: 1 sample was taken from the tumor-draining mesenteric vein, 1 sample was obtained through a central venous catheter from the superior vena cava, and 1 sample was taken from the antecubital vein. After resection of the tumor, another central venous blood sample was obtained.

All samples were diluted with 10 mL of phosphate-buffered saline. After density centrifugation through Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) (30 minutes, 400g), mononuclear cells were harvested from the interphase and washed twice in phosphate-buffered saline. The cell pellet was then shock frozen in liquid nitrogen at −70°C.

METHODS

Total RNA from mononuclear blood cells was extracted as previously described.15

The CK 20 RT-PCR was performed as previously described.15 Briefly, complementary DNA was reverse-transcribed with a commercial kit (Life Technologies Gibco BRL, Karlsruhe, Germany), with the use of 1 µg of RNA as recommended by the manufacturer and a specific oligonucleotide (CK 20558.rev) in a total reaction volume of 20 µL. The PCR was performed under the following conditions: 25 pmol of each primer, 0.2 mmol of 2'-deoxynucleoside 5'-triphosphate (dNTP) per liter, 1.5 mmol of magnesium chloride per liter, 2.5 U of Taq DNA polymerase, and PCR buffer: 20 mmol of Tris-hydrochloride per liter and 50 mmol of potassium chloride per liter (Life Technologies Gibco BRL). For the first PCR, 5 µL of the RT reaction mixture was used to amplify CK 20 complementary DNAs in a total reaction volume of 100 µL with the use of primer 1.for (ATG GAT TTC AGT CGC AGA) and primer 558.rev (ATG TAG GGT TAG GTC ATC AAA G). Thirty-five rounds of amplification were performed at 30-second intervals at temperatures of 93°C, 60°C, and 72°C, with a final extension step of 6 minutes. Twenty microliters of this reaction mixture was then subjected to the nested PCR with a total reaction volume of 100 µL and primers 139.for (TCC AAC TTC AGA CAC ACG GTG AAC TAT G) and 429.rev (CAG GAC ACA CGG AGC ATT TGG CAG) (35 cycles, 30 seconds at 93°C, 30 seconds at 72°C, and 30 seconds at 72°C; final extension step, 10 minutes).

The PCR products were analyzed by electrophoresis on 2% agarose gels (Figure). The CK 20 PCR products were blotted onto nylon membranes (Hybond N+; Amersham Life Science, Buckinghamshire, England) and hybridized with a chemoluminescence-labeled oligonucleotide probe (ECL Detection System; Amersham Life Science) consisting of nucleotides 5269 to 5280 on exon 1 and nucleotides 7429 to 7448 on exon 2 of the CK 20 sequence (CTG CGA AGT CAG ATT AAG GAT G).

The RNA quality and performance of reverse transcription of all analyzed samples were confirmed by RT-PCR amplification of glyceraldehyde phosphate dehydrogenase transcripts as previously described.16

The sensitivity of the CK 20 RT-PCR assay was evaluated in a previous study15 and reproducibly allowed the detection of about 10 colon cancer cells (HT 29) in 10 mL of blood.

SUBJECTS

Informed consent was obtained from all patients. The study protocol was approved by the ethics committee of the University of Heidelberg, Heidelberg, Germany.

The study included 40 patients treated at the Department of Surgery, University of Heidelberg (21 men and 19 women, aged 36-78 years; median age, 62.5 years). All patients had histologically confirmed colorectal adenocarcinoma and were undergoing curative (R0) resection according to the “no-touch isolation” technique19,20 with systematic regional lymph node dissection. Tumor stage and grading were classified according to the fifth edition of the TNM classification of the International Union Against Cancer.21

The control group consisted of peripheral blood samples from 34 healthy volunteers and from 14 patients with benign colorectal disease. In addition, central venous blood samples (taken preoperatively, intraoperatively, and postoperatively) from 18 patients and mesenteric venous blood samples from 10 patients undergoing colorectal resection for benign diseases (sigmoid diverticulosis, Crohn disease, and ulcerative colitis) served as negative controls.

STATISTICAL ANALYSIS

Statistical computations were performed with the software package StatXact4 for Windows (Cytel Software Corp, Cambridge, Mass). Results were considered statistically significant at P<.05.

The McNemar and Pearson χ² tests22 were used to test for differences between the detection rates in mesenteric, central, and peripheral venous blood.

The Cochran-Armitage trend test22 was used to examine the relationship between tumor stage and detection of circulating tumor cells in mesenteric venous blood.
The lower stages, although tumor cells could also be detected in mesenteric venous blood of a substantial number of patients with stages I and II (5/12 [42%] and 5/11 [45%], respectively).

The comparison between tumor cell detection in mesenteric venous blood and central venous blood (taken at the same time) is shown in the Table. There was a clear relationship between tumor cell detection in central venous blood and mesenteric venous blood: 5 of 6 patients with tumor cells in central venous blood also tested positive for CK 20 in mesenteric venous blood. Only 1 of 6 patients with tumor cells in central venous blood did not show tumor cells in mesenteric venous blood.

In central venous blood obtained after tumor resection, tumor cells were detected in 11 (28%) of 40 patients compared with 6 (15%) of 40 patients before tumor resection, indicating an enhanced release of tumor cells during surgery for colorectal cancer.

Nineteen of 40 patients with positive CK 20 RT-PCR results in central venous blood samples obtained after tumor resection also showed tumor cells in their mesenteric venous blood samples (taken before tumor resection).

In this study, we investigated the frequency of circulating tumor cells in mesenteric venous blood samples compared with central and peripheral venous blood samples of 40 patients undergoing resection of colorectal cancer. Isolated disseminated tumor cells were detected by CK 20 RT-PCR in mesenteric venous blood samples of 20 (50%) of 40 patients with colorectal cancer.

Previous studies using conventional cytologic examination or immunocytochemistry for the detection of colorectal cancer cells in mesenteric venous blood observed detection rates between 14% and 57%. Using immunocytochemistry, Leather et al demonstrated tumor cells in only 14% of mesenteric venous blood samples of patients with colorectal cancer. The varying incidence of circulating tumor cells in mesenteric venous blood samples reported in the literature may be due to different sampling times (eg, before and after tumor mobilization) and low sensitivity and specificity of the detection methods used. The prognostic impact of these results is controversial. Recently, PCR-based protocols were introduced to further increase sensitivity and specificity of detection of colorectal cancer cells in mesenteric venous blood. Using a carcinoembryonic antigen RT-PCR, Ueda et al detected cancer cells in mesenteric venous blood of 11 (42%) of 26 patients with resectable tumors.

Local tumor-draining mesenteric vein (before passage of released tumor cells through liver capillary vessels).

In a prospective study we analyzed mesenteric and central venous blood samples compared with central and peripheral venous blood samples obtained at the same time.

**RESULTS**

All blood samples from the above-defined control group consistently tested negative for CK 20 expression. The blood samples all showed a positive glyceraldehyde phosphate dehydrogenase amplification signal, confirming the quality of RNA and adequate performance of reverse transcription. The primary tumor samples all tested positive for CK 20 messenger RNA expression.

In a prospective study we analyzed mesenteric and central venous blood samples from 40 patients and peripheral venous blood samples from 19 patients undergoing resection of colorectal cancer. Peripheral venous blood was collected from only 19 patients because it is technically difficult to obtain peripheral venous blood samples intraoperatively. After tumor resection, central venous blood samples were again obtained from all 40 patients.

Tumor cell detection in blood drawn simultaneously before tumor resection of 40 patients with colorectal cancer was stratified according to the different blood compartments. There was a statistically significantly higher detection rate (P<.001) in mesenteric venous blood samples (20/40 [50%]) compared with central (6/40 [15%]) and peripheral (2/19 [11%]) venous blood samples.

The detection rate of colorectal cancer cells in mesenteric venous blood was increased in stages III and IV (7/13 [54%] and 3/4 [75%], respectively) compared with the lower stages, although tumor cells could also be detected in mesenteric venous blood of a substantial number of patients with stages I and II (5/12 [42%] and 5/11 [45%], respectively).

The comparison between tumor cell detection in mesenteric venous blood and central venous blood (taken at the same time) is shown in the Table. There was a clear relationship between tumor cell detection in central venous blood and mesenteric venous blood: 5 of 6 patients with tumor cells in central venous blood also tested positive for CK 20 in mesenteric venous blood. Only 1 of 6 patients with tumor cells in central venous blood did not show tumor cells in mesenteric venous blood.

In central venous blood obtained after tumor resection, tumor cells were detected in 11 (28%) of 40 patients compared with 6 (15%) of 40 patients before tumor resection, indicating an enhanced release of tumor cells during surgery for colorectal cancer.

Nine of 11 patients who had positive CK 20 RT-PCR results in central venous blood samples obtained after tumor resection also showed tumor cells in their mesenteric venous blood samples (taken before tumor resection).

**COMMENT**

In this study, we investigated the frequency of circulating tumor cells in mesenteric venous blood samples compared with central and peripheral venous blood samples of 40 patients undergoing resection of colorectal cancer. Isolated disseminated tumor cells were detected by CK 20 RT-PCR in mesenteric venous blood samples of 20 (50%) of 40 patients with colorectal cancer.

Previous studies using conventional cytologic examination or immunocytochemistry for the detection of colorectal cancer cells in mesenteric venous blood observed detection rates between 14% and 57%. Using immunocytochemistry, Leather et al demonstrated tumor cells in only 14% of mesenteric venous blood samples of patients with colorectal cancer. The varying incidence of circulating tumor cells in mesenteric venous blood samples reported in the literature may be due to different sampling times (eg, before and after tumor mobilization) and low sensitivity and specificity of the detection methods used. The prognostic impact of these results is controversial. Recently, PCR-based protocols were introduced to further increase sensitivity and specificity of detection of colorectal cancer cells in mesenteric venous blood. Using a carcinoembryonic antigen RT-PCR, Ueda et al detected cancer cells in mesenteric venous blood of 11 (42%) of 26 patients with resectable tumors.
colon cancer. However, carcinoembryonic antigen RT-PCR may yield false-positive results in peripheral blood.\textsuperscript{28,29} Specificity was not analyzed in these studies, because mesenteric venous blood samples from patients with benign gastrointestinal diseases were not available to serve as negative controls.

We have used a sensitive and specific CK 20 RT-PCR system for detection of colon cancer cells in different blood compartments. Focusing on mesenteric venous blood in the present analysis, specificity of our detection method was confirmed by examining mesenteric venous blood samples from patients with benign gastrointestinal diseases. Mesenteric venous blood samples from 10 patients and central venous blood samples (taken preoperatively, intraoperatively, and postoperatively) from 18 patients undergoing surgery for benign colorectal diseases all tested negative for CK 20 expression. This suggests that either no exfoliated normal colon epithelial cells expressing CK 20 were released into central or portal venous blood during surgery or, more likely, that these exfoliated normal colon cells rapidly underwent apoptosis by loss of adherence\textsuperscript{30} after having been released into the blood circulation.

The frequent event of circulating tumor cells in mesenteric venous blood of patients with colorectal cancer, even in early stages of cancer, supports the surgical concept of the no-touch isolation technique described by Barnes\textsuperscript{30} and by Turnbull et al\textsuperscript{10} with initial lymphovascular ligation before resection of colorectal cancer. A small, nonsignificant survival benefit was observed in a randomized trial comparing the no-touch isolation technique with conventional colorectal resection.\textsuperscript{31} However, with use of the mutant allele specific amplification method, Hayashi et al\textsuperscript{32} recently reported that the no-touch–isolation technique reduced intraoperative shedding of tumor cells into portal venous blood during resection of colorectal cancer.

The cascade theory assumes that hematogenic metastases from primary colorectal cancer develop in a defined stepwise progression, with anatomic drainage of the cancer cells via portal venous blood into the liver as the first site of metastasis and from there into the lungs and systemic circulation.\textsuperscript{32} Indeed, it was shown in an animal study that hepatic metastasis depends on hepatic first-pass trapping of colon cancer cells\textsuperscript{33}; however, only a small percentage of these tumor cells develop into clinically overt metastasis.\textsuperscript{34} If this cascade theory represents the main pattern of hematogenic metastasis in colorectal cancer, one would expect that all or nearly all circulating tumor cells in portal venous blood are trapped in the liver. To determine the extent of tumor cell spread in venous blood after passage of the tumor cells through the liver, we examined central and peripheral venous blood samples taken simultaneously with mesenteric venous blood samples. The significantly higher detection rate of tumor cells in mesenteric venous blood compared with central and peripheral venous blood indicates that the liver and other organs (eg, lungs and peripheral capillary system) have an important filter function for circulating tumor cells in patients with colorectal cancer. On the other hand, the detection of circulating tumor cells in central and peripheral venous blood supports the concept of early systemic hematogenic dissemination of colorectal cancer. It also confirms that the filtering effect of the liver for colorectal cancer cells is not absolute in some patients. Interestingly, there is a clear relationship between detection of colorectal cancer cells in mesenteric venous blood and tumor cell detection in central venous blood. Our data indicate that both theories on hematogenic tumor cell dissemination play an important role in the process of hematogenic colorectal cancer metastasis.

After resection of the tumor, we obtained a second central venous blood sample to detect intraoperative tumor cell dissemination. In central venous blood taken immediately after tumor resection, we detected tumor cells in 11 (28%) of 40 patients compared with 6 (15%) of 40 patients before tumor resection. This confirms the results of our previous study\textsuperscript{15} in which a significantly enhanced release of tumor cells into central venous blood during surgery for colorectal cancer was observed. Recently, we showed that intraoperative tumor cell dissemination was detected more frequently in patients undergoing resection of liver metastases of colorectal cancer than in patients with resection of primary colorectal cancer.\textsuperscript{17}

Although the development of liver metastasis requires a series of sequential, interrelated steps,\textsuperscript{39} the existence of colorectal cancer cells in mesenteric and central venous blood is thought to be a risk factor for liver metastases. However, the high incidence of colorectal cancer cells in mesenteric and central venous blood suggests that a perioperative or postoperative specific therapy (eg, with monoclonal antibodies) against circulating colorectal cancer cells might be an effective tool in adjuvant therapy. This potential therapeutic effect could be monitored by CK 20 RT-PCR. Clinical follow-up of our patients will provide data on the prognostic significance of disseminated colorectal cancer cells in mesenteric, central, and peripheral venous blood. A prognostic significance of detection of disseminated tumor cells in bone marrow of patients with colorectal cancer has already been demonstrated.\textsuperscript{37}

In conclusion, the ability of CK 20 RT-PCR to detect a very small number of tumor cells in mesenteric, central, and peripheral venous blood is likely to provide the clinician with a new diagnostic tool. Detailed analysis may define subgroups of patients at high risk for metastatic disease from colorectal cancer who could benefit from new adjuvant therapy regimens.

Reprints: Magnus von Knebel Doeberitz, MD, Division for Molecular Diagnostics and Therapy, Department of Surgery, University of Heidelberg, INF 110, D-69120 Heidelberg, Germany (e-mail: knedel@med.uni-heidelberg.de).

REFERENCES


Surgical Anatomy

Meckel’s diverticulum is a persistence of the vitelline duct (omphalomesenteric duct), creating an ileal diverticulum, usually on the antimesenteric border. It is within 2 ft of ileocecal valve, present in 2% of the population, and 2% are symptomatic.