Dissemination of Tumor Cells in Patients Undergoing Surgery for Colorectal Cancer

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ABSTRACT
The majority of patients with colorectal cancer present at a stage when the primary cancer can be resected with curative intent. However, despite the high resectability rate, about 30–50% of these patients subsequently develop metastatic disease. In these patients, neoplastic cells were disseminated either before or during surgery of the primary cancer. Due to the lack of appropriate detection systems, the extent of pre- and intraoperative hematogenic tumor cell dissemination has not yet been determined. Using a reverse transcription-PCR assay to amplify cytokeratin 20 transcripts, we were able to detect 10 colorectal cancer cells in 10 ml of blood. Blood samples were taken from 65 patients undergoing resection of primary colorectal cancer or liver metastasis of colorectal cancer pre-, intra-, and postoperatively. Circulating tumor cells were detected in 24 of 58 patients with colorectal resections in correlation to the tumor stage and in 6 of 7 patients who underwent hemihepatectomy for liver metastasis. In 8 of 58 patients with colorectal resection and in 5 of 7 patients with hemihepatectomy, tumor cells could only be detected during or during and after surgery. These results demonstrate that hematogenic tumor cell dissemination is a frequent and early event in colorectal cancer. Surgery enhances the release of tumor cells into the circulation. The long-term follow-up of our patient cohort will provide data on the prognostic relevance of circulating tumor cells and might lead to new therapeutic concepts for perioperative prophylaxis of tumor cell implantation or postoperative adjuvant therapy regimens.

INTRODUCTION
About 30–50% of patients with colorectal cancer initially presenting with resectable tumors subsequently develop metastatic disease (1). Therefore, dissemination of metastatic tumor cells is likely to be an early event in tumor progression that may happen pre- or intraoperatively and is not detected by conventional staging techniques. Although the exact relationship between circulating tumor cells and the development of recurrent disease in colorectal cancer is not yet understood, it is generally assumed that enhanced dissemination of cancer cells before or during surgery contributes significantly to the development of metastatic disease.

Previous studies focused on the detection of disseminated colorectal cancer cells in bone marrow aspirates using immunocytological methods (2, 3). In about 32% of patients, disseminated tumor cells were detected in bone marrow aspirates, and detection of tumor cells in bone marrow was clearly related to an earlier relapse and decreased survival of the respective patients (4). This data initiated a new therapeutic concept using monoclonal antibodies directed against a specific antigen expressed on colorectal epithelia for patients with stage III colorectal cancer as an adjuvant treatment (5).

Tumor cells were also detected in peripheral blood samples of patients with colorectal carcinomas, but their prognostic impact has been controversial (6–8). Using conventional cyto logical examination of blood smears, the incidence of circulating tumor cells during resection for colorectal cancers has been reported to vary from 25–67% (9–11). The variability in these reports may be the result of the low sensitivity and specificity of cytological blood smear analysis for tumor cell detection. More recently, immunohistochemistry was used to detect disseminated tumor cells in peripheral blood samples. This study revealed evidence for circulating tumor cells in 4 of 42 patients with colorectal carcinoma (12).

PCR-based protocols have improved the sensitivity and specificity of detection systems for disseminated cancer cells, allowing the identification of approximately 1 malignant cell in 10^7 normal peripheral mononuclear blood cells (13). RT-PCR 2 amplification of tissue-specific mRNAs is an appropriate technique for the detection of disseminated colorectal cancer cells. CK-20 transcripts seem to be good targets, because they are expressed in gastrointestinal epithelia, urothelium or Merkel cells, and derived tumors, but not in other nontransformed tissues (14, 15). Burchill et al. (16) described a CK-20 RT-PCR system for the detection of malignant colon epithelia in blood and bone marrow samples, and Soeth et al. (17) used this assay to detect disseminated cancer cells in bone marrow aspirates of gastrointestinal cancer patients. Their study suggested a high
correlation between the prevalence of tumor cells detected by amplification of CK-20 mRNAs in bone marrow samples and advanced disease. Gunn et al. (18) examined the bone marrow and lymph nodes of patients with colorectal cancer with a CK-20 RT-PCR assay. Their detection rate was low, possibly due to a low sensitivity of the used PCR.

Surgical manipulation of tumors always bears the risk of intraoperative tumor cell dissemination. Animal studies suggested that manipulation of tumors leads to tumor cell shedding into the blood stream (19, 20) with an increased occurrence of distant metastasis (21, 22). To prevent intraoperative tumor cell dissemination, Turnbull et al. (23) described the “no-touch isolation” technique with initial lymphovascular ligation for resection of colorectal cancer. They demonstrated an improved survival rate with this technique. This improved prognosis, however, could not be confirmed in other studies (24).

Thus, the extent and prognostic significance of pre- and intraoperative tumor cell dissemination in human colorectal cancer has not been determined adequately. The purpose of this study was the development of a sensitive and specific assay for the detection of colorectal cancer cells in peripheral blood samples to determine the extent of pre- and intraoperative tumor cell dissemination.

PATIENTS AND METHODS

Blood Samples

Blood samples (10 ml) were obtained through a central venous catheter in the vena cava superior and diluted with 10 ml of PBS. After density centrifugation through Ficoll-Paque (Pharmacia; 30 min, 400Xg), mononuclear peripheral blood cells were harvested from the interphase and washed twice in PBS. After density centrifugation through Ficoll-Paque (Pharmacia; 30 mm, 400Xg), mononuclear peripheral blood cells were harvested from the interphase and washed twice in PBS. The cell pellet was then shock-frozen in liquid nitrogen and stored at -70°C.

RNA Extraction

RNA from cell lines, peripheral mononuclear blood cells, and frozen tissue sections of tumors were extracted using a commercially available RNA extraction kit (Glassmax; Life Technologies, Inc., Karlsruhe, Germany) in accordance with the recommendations of the manufacturer. To eliminate contaminating DNA within the RNA preparation, 1 µg of RNA was digested with RNase-free DNase I, as recommended by the supplier (Life Technologies, Inc.; 15 min, 25°C).

RT-PCR

Sequences for oligonucleotide primers in the CK-20 cDNA sequence were identified using the Heidelberg Unix Sequence Analysis Software Program. To increase the sensitivity and specificity, we developed a nested PCR protocol. Primers located on exons 1 and 3 and exons 1 and 2 were selected to allow the differentiation of amplified spliced transcripts and contaminating genomic DNA because of their different sizes (Fig. 1). For the first PCR reaction, primers (1.for and 558.rev) with an annealing temperature approximately 10°C below that of the primers of the nested PCR (139.for and 429.rev) were chosen to prevent binding of the outer primers to amplification products in nested PCR reactions. The optimal annealing temperatures for both primer pairs were tested experimentally using various annealing temperatures.

The cDNA was synthesized with a reverse transcription kit (Life Technologies, Inc.), using 1 µg of RNA as recommended by the manufacturer and the primer CK-20 558.rev in a total reaction volume of 20 µl.

The PCR was performed using the following reaction conditions: primers, 25 pmoles each; deoxynucleotide triphosphate, 0.2 mm each; MgCl₂, 1.5 mm; Taq DNA polymerase, 2.5 units; and PCR buffer, 20 mm Tris-HCl and 50 mm KCl (PCR kit; Life Technologies, Inc.). For the first PCR, 5 µl of the reverse transcription reaction mixture were used to amplify CK-20 cDNAs in a total reaction volume of 100 µl using the primers 1.for (ATGGATTCAGTCGCGAGA) and 558.rev (ATGGATGGTATGCATCAAGA). Thirty-five rounds of amplification were performed at 30-s intervals at temperatures of 93°C, 60°C, and 72°C, with a final extension step of 10 min.

Twenty µl of this reaction mixture were then subjected to the nested PCR. The nested PCR reaction was performed in a total volume of 100 µl with primer 139.for (TCCAACCTCCAGACACGCGTTGAATCTAGT) and 429.rev (CAGGACACCGAGCAATTTGCCAG) (35 cycles, 30 s at 93°C, 30 s at 72°C, and 30 s at 72°C, with a final extension step of 10 min). PCR products were analyzed by electrophoresis on 2% agarose gels. In addition, direct thermocycle sequencing of the obtained amplimer was performed.

RNA quality and performance of reverse transcription of all analyzed samples were confirmed by RT-PCR amplification of glyceraldehyde-3-phosphate dehydrogenase transcripts as described previously (25).

Sensitivity of CK-20 RT-PCR

The sensitivity of the CK-20 RT-PCR assay was determined in dilution experiments using the colon cancer cell line HT 29 (26). Tumor cells (0, 10, 10², and 10³) were added to 10 ml of peripheral blood samples of healthy donors, and these blood samples were then analyzed by CK-20 RT-PCR.

Sequencing of PCR Products

CK-20 PCR products were cloned into pCR2.1 vector (TA cloning kit; Invitrogen, San Diego, CA) after plasmid DNA

![Schematic illustration of the CK-20 gene. derived mRNA/cDNA, and CK-20 RT-PCR products. The relative position of the primers used for the RT-PCR is indicated by arrows. (E: exon; bp, base pairs: 1.PCR, first PCR; 2.PCR, nested PCR.](image-url)
preparation sequencing was performed, using a Cy5'-AutoRead sequencing kit (Pharmacia Biotech, Freiburg, Germany). Sequencing reactions were analyzed on denaturing 6.6% polyacrylamide/7 M urea gels using an ALFExpress DNA sequencer (Pharmacia Biotech).

Patients

Informed consent was obtained from all patients, and the study protocol was approved by the ethics committee of the University of Heidelberg. Sixty-five patients treated at the Department of Surgery, University of Heidelberg (37 males and 28 females; ages, 44–85 years; mean age, 65.2 years) from April to September 1996 were included: 58 patients with histologically confirmed colorectal adenocarcinoma (29 patients with rectal carcinoma and 29 patients with colon carcinoma) undergoing curative (R0) resection according to the no-touch isolation technique; and 7 patients with liver resection for liver metastasis of colorectal carcinoma. Three blood samples were obtained from each patient through a central venous catheter: the first was obtained after induction of anesthesia, the second was obtained after resection of the tumor, and the third was obtained 24 h after the operation. Intraoperative blood loss and substitution with plasma and erythrocytes were recorded. Tumor stage and grading were classified according to the 4th edition of the tumor-node-metastasis classification of the International Union Against Cancer (27).

Blood samples from 18 patients undergoing colorectal resection and 6 patients undergoing liver resection for benign diseases were obtained before, during, and 24 h after surgery as controls. In addition, peripheral blood samples from 12 healthy volunteers were analyzed.

Statistical Analysis

Statistical computations were done using the software packages S-PLUS Version 3.4 (MathSoft, Inc.) and StatXact3 for Windows (Cytel Software Corp.). A result was always judged as statistically significant if the P of its respective test statistic was less than or equal to 5% (P ≤ 0.05).

Tumor, Node, and Stage versus PCR Results. The Cochran-Armitage trend test (28) was used to determine the relationship between tumor, node, and stage and the detection of circulating tumor cells. This test is used to determine if the series of four observed subclasses (PCR results for T1–4, N0–3, and Stages I–IV) all have the same underlying binomial response rate, or, if the rates are unequal, either increasing or decreasing in order.

PCR Results versus Timing of Blood Collection. A logistic regression analysis (29) was performed to analyze the relationship between tumor cell detection and intraoperative blood loss. A possible cutoff point of intraoperative blood loss for tumor cell detection was determined using the maximally selected $\chi^2$ statistic (30) with P correction according to Lausen and Schumacher (31). To correlate the PCR results with the timing of blood collection, Cochran’s Q test (32) was performed. This test was constructed to find systematic changes in the distribution of the PCR results over all three time points of blood collection. For evaluation of intraoperative tumor cell dissemination, we performed a partitioned $Q:Q_{\text{adj}}$ (32), intraoperative versus pre- or post-operative.

RESULTS

Sensitivity of CK-20 RT-PCR

A 290-bp PCR product was amplified from samples enriched with HT 29 cells up to a dilution of 10 HT 29 cells in 10 ml of peripheral blood samples of a healthy donor. W, water negative control; M, molecular weight marker; P, positive control (HT 29 cells).

Specificity of CK-20 RT-PCR

Blood samples from 12 healthy volunteers consistently tested negative for CK-20 expression. To exclude the possibility of exfoliation of CK-20-expressing normal colon mucosa cells or liver cells during surgical manipulation, blood samples from 18 patients undergoing colorectal resection and 6 patients undergoing liver resection for benign disease were taken pre-, intra-, and postoperatively and screened for expression of CK-20. None of these revealed a detectable PCR product. To prove CK-20 expression in colorectal carcinomas, 20 primary tumor samples and 10 samples of colorectal liver metastasis were analyzed. All of these tested positive for CK-20 mRNA expression.

Patient Study

Correlation between Tumor Cell Detection and Tumor Stage. CK-20 transcripts were detected in at least one of the three blood samples taken before, during, and after surgery in 24 of 58 patients (41%) undergoing resection of primary colorectal carcinomas, in a statistically significant stage-dependent manner (Table 1).

Correlation between Tumor Cell Detection and Timing of Blood Collection. In 9 patients (1 of 19 stage II patients, 3 of 16 stage III patients, and 5 of 12 stage IV patients), tumor cells were detected pre-, intra-, and postoperatively. In 8 patients, tumor cells were only detected during or during and after surgery (4 of 19 stage II patients, 1 of 16 stage III patients, and 3 of 12 stage IV patients). In 6 patients, tumor cells could only
be detected preoperatively (1 of 11 stage I patients, 1 of 19 stage II patients, 2 of 16 stage III patients, and 2 of 12 stage IV patients). In one patient, CK-20 expression could only be demonstrated postoperatively (Table 2).

A logistic regression analysis of pre-, intra-, and postoperative tumor cell detection depending on the amount of blood loss was done to assess the influence of intraoperative blood loss on intraoperative tumor cell detection by CK-20 RT-PCR. The Ps for preoperative and for postoperative tumor cell detection were $P = 0.85$ and $P = 0.99$, respectively. The $P$ for intraoperative detection was $P = 0.07$, with an estimated odds ratio of 0.5, with a 95% confidence interval of 0.26–1.05 for a 0.5 liter increase in blood loss. Thus, the chance of intraoperative tumor cell detection is halved by an increase in blood loss of 0.5 liter. We conclude that the amount of blood loss seems to be relevant with a 95% confidence interval of 0.26–1.05 for a 0.5 liter increase in blood loss. Consequently, patients with an intraoperative blood loss of more than 1 liter were excluded maximally selected for intraoperative tumor cell detection. Consequently, the detection of a cutoff point of intraoperative blood loss was done to assess the influence of intraoperative blood loss for intraoperative tumor cell detection depending on intraoperative blood loss (Table 4). Cochran’s $Q$ test revealed a statistically significant association between the PCR results and the timing of blood collection ($P = 0.039$). The special question of intraoperative tumor cell dissemination was tested by a partitioned $Q:Q_{	ext{def}}$, showing a statistically significant increase of tumor cell detection intraoperatively ($P = 0.007$).

Six of seven patients with liver resection for hepatic metastasis of colorectal cancers tested positive for CK-20-expressing cells in peripheral blood samples. In two of seven patients, tumor cells were detected only intraoperatively, and in three of seven patients, tumor cells were detected intra- and postoperatively. One patient displayed CK-20-expressing cells in blood samples obtained before, during, and after surgery (Fig. 3).

### DISCUSSION

In this study, we investigated the rate of tumor cell dissemination in the peripheral circulation of 65 patients with colorectal carcinomas using a CK-20 RT-PCR assay for the detection of circulating tumor cells. The sensitivity of the system was evaluated in tumor cell dilution experiments and reproducibly allowed the detection of about 10 tumor cells in 10 ml of blood.

Specificity is a major concern in RT-PCR systems for the detection of disseminated tumor cells (13). Obviously, the target gene must not be expressed in normal peripheral blood cells. False positive results can be caused by contamination, illegitimate transcription, or pseudogenes. Specificity of the used CK-20 RT-PCR was ensured by the following measures: (a) selection of a target gene (CK-20) without expression in peripheral blood cells and without known pseudogenes; (b) location of PCR primers on exons 1 and 3 and exons 1 and 2, respectively, to allow the differentiation of amplified spliced transcripts and contaminating genomic DNA because of their different sizes (Fig. 1); (c) treatment of RNA samples with DNase to remove all contaminating DNA. Blood samples of 12 healthy individuals consistently tested negative with the established CK-20 RT-PCR. Twenty-four patients undergoing surgery for nonneoplastic colorectal or liver disease also tested negative, suggesting that either no CK-20-expressing colorectal epithelia or liver cells were released into the circulation during surgery or, more likely, that normal colon epithelia or liver cells rapidly underwent anaplasia (33) after having been released into the circulation.

Circulating tumor cells were detected by CK-20 RT-PCR in 41% of colorectal cancer patients undergoing colorectal surgery and six of seven patients undergoing hemihepatectomy for liver metastasis. As expected, circulating tumor cells were detected significantly more frequently in patients with advanced disease.

Various experimental studies in animal cancer models suggested that surgical manipulation of malignant tumors might
significantly contribute to tumor cell dissemination (19-22). Turnbull et al. (23) described the no-touch isolation technique for resection of colorectal cancer to prevent intraoperative tumor cell dissemination. However, his technique is not generally accepted, due to contradictory experimental and clinical results (24).

To detect intraoperative tumor cell dissemination, blood samples were drawn pre-, intra-, and postoperatively. The association of PCR results and timing of blood collection was evaluated statistically: in nine patients, tumor cells were detected pre-, intra-, and postoperatively. In eight patients, tumor cells were only detected during or during and after surgery, possible evidence for tumor cell shedding due to surgical manipulation. In six patients, however, tumor cells could only be detected preoperatively, and in one patient, CK-20 expression could only be demonstrated postoperatively. The unexpected results of the latter seven patients could be due to false negative results, because of unavoidable sampling errors. However, this could also be explained by a dilution effect of tumor cells due to intraoperative blood loss and transfusion, because five of these seven patients had high intraoperative blood losses (>1.5 liter). A logistic regression analysis revealed that the chance of intraoperative tumor cell detection is halved by an increase in blood loss of 0.5 liter. We were able to define 1 liter as the cutoff point of intraoperative blood loss for reliable tumor cell detection using the CK-20 RT-PCR. Consequently, patients with an intraoperative blood loss of more than 1 liter were excluded from statistical evaluation of the association between PCR results and the timing of blood collection. We found a statistically significant correlation between the PCR results and the timing of blood collection and a statistically significant increase of tumor cell detection intraoperatively. Thus, our results demonstrated intraoperative tumor cell dissemination in 8 of 43 patients (19%) with an intraoperative blood loss of less than 1 liter, corresponding to 8 of 32 patients (25%) in whom no circulating tumor cells were observed in blood samples taken before surgery.

In five of seven patients who underwent hemihepatectomy for liver metastasis due to colorectal cancers, disseminated tumor cells were only detected during or during and after surgery, but not before surgery. The more frequent detection of intraoperative tumor cell dissemination in patients undergoing hemihepatectomy compared to the patients with colorectal resections could be due to the technical impossibility of an early occlusion of venous drainage during resection in the former group. However, the no-touch isolation technique does not completely prevent intraoperative tumor cell dissemination. Surgical manipulation of malignant tumors seems to contribute to tumor cell dissemination.

In this study, we detected tumor cells in blood samples obtained through a venous catheter in the vena cava superior. Cells derived from colorectal epithelium have to pass through the capillary vessels in the liver, the lung, and the upper half of the body before they reach the vena cava superior. Thus, tumor cells released during surgery and detected in our study have passed at least three capillary vessel systems. How many neoplastic cells were retained in the capillary systems of the liver or the lungs will be analyzed in biopsy samples in forthcoming studies.

The presence of circulating tumor cells does not necessarily predict subsequent metastatic disease. The implantation of circulating tumor cells seems to be very inefficient, and circulating tumor cells might be destroyed rapidly (34-37). However, the activation of blood coagulation during surgery, which may lead to an increased entrapment of circulating tumor cells and the relative immune suppression caused by surgical stress, could enhance the metastatic potential of circulating tumor cells (38). In addition, the metastatic potential of individual tumor cells might differ significantly (39). Therefore, the prognostic significance of circulating tumor cells in patients with colorectal cancer is still unclear. However, the increased frequency of disseminated tumor cells in patients with advanced stages of the disease in the present study and the well-known observation that patients with locally advanced disease tend to develop metastasis more frequently although the primary cancer has been resected curatively suggest an increased risk of metastasis for patients with disseminated neoplastic cells. This study reveals, that pre- and intraoperative tumor cell dissemination is a frequent event in colorectal cancer. The long-term follow-up of our patient cohort will now provide data on the prognostic relevance.
of circulating tumor cells detected by CK-20 RT-PCR amplification, with potential implications for adjuvant therapy regimens.

The unexpected high frequency of circulating tumor cells released during surgery despite application of the no-touch isolation technique suggests that a perioperative prophylaxis of tumor cell implantation might be effective in preventing metastatic relapse in patients undergoing curative surgery for colorectal cancers. This prophylaxis could presumably be achieved by high titer antibodies directed against surface molecules on disseminated tumor cells administered perioperatively, which has to be evaluated in additional studies.

REFERENCES


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