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ORIGINAL ARTICLE

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# Peri-operative filtration of disseminated cytokeratin positive cells in patients with colorectal liver metastasis

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**Abstract** *Background:* Liver resection is the only potential cure for patients with colorectal liver metastasis. However, more than 30% of patients will develop tumour recurrence, probably caused by tumour cells disseminated before or during surgery. As prevention of cell dissemination is barely obtainable, alternative concepts have to be discussed. *Methods:* The potential of leukocyte adhesion filters for the removal of cytokeratin positive cells (CK+) from blood was studied in 18 patients undergoing liver resection for colorectal liver metastasis. Blood sampling was done via a liver venous catheter during hepatic mobilisation. Filtration was done with an in-line WBF2 filter system. To define the relation between surgery and cell release we compared patients' pre-operative and intra-operative blood and bone marrow (BM) samples with their CK expression using immunochemical staining. *Results:* CK+ cells were detected in BM samples of nine of 14 patients before surgery, indicating early dissemination. In ten of 18 patients CK+ cells were detected in blood samples during hepatic mobilisation; all ten patients underwent major liver surgery (R0 resection). In those patients recurrent disease was observed more often ($P \leq 0.05$). In 17 of 18 patients CK+ cells were not detectable after filtration procedure, which indicated cell adhesion to the filter medium. *Conclusion:* Liver resection due to metastasis leads to frequent intra-operative tumour cell shedding. As the detection of CK+ cells is correlated with disease recurrence, modification of surgical techniques to prevent cell dissemination, and additional therapeutic concepts such as advanced filtration technology, have to be discussed.

**Keywords** Tumour cell dissemination · Liver surgery · Liver venous catheter · Cell filtration

## Introduction

The concept of preventing intra-operative tumour cell dissemination was already described in the 1960s by the so-called no-touch bowel surgery, demanding closure of lymph–vascular structures prior to preparation and resection of primary colorectal cancer [1]. However, despite these surgical principles liver metastasis still occurs in 20%–40% of patients, after potential curative resection of colorectal malignancies, as a result of portal venous tumour cell dissemination prior to or during operation [2]. In contrast to colorectal surgery, prevention of intra-operative tumour cell dissemination is difficult to obtain in patients who are undergoing liver resection for primary and secondary malignancies. This was confirmed by Weitz et al., who recently reported that cytokeratin-20 reverse transcriptase–polymerase chain reaction (RT-PCR) transcripts in patients who were suffering from colorectal liver metastases were detected significantly more often in central venous blood samples during surgery than before and after surgery. Weitz et al. concluded that liver resection implements an increased risk for intra-

operative tumour cell dissemination [3]. The high incidence of mechanically induced intra-operative tumour cell dissemination might be caused by anatomical reasons and the need for extensive mobilisation before feasible occlusion of the venous drainage [4]. Surgical mobilisation is almost unpreventable in commonly used resection techniques, especially for tumours located beneath the hepatic veins.

We recently demonstrated in an unpublished pilot study of 48 patients with colorectal liver metastases that, although cytokeratin positive (CK+) cells were often detected in bone marrow before surgery, additional cell dissemination was frequently observed in liver venous blood during hepatic mobilisation. Methods to reduce this tumour cell release into the systemic circulation are rare. As shown in previous studies, some filter media used for leukocyte depletion are able to remove tumour cells under in vitro conditions [5, 6]. Since safe and differentiated placement of catheters into selected liver veins is possible [7], cell filtration, using special cell adhesion filters within the blood drained from major veins located close to the tumour, could be a useful and safe [8] additional therapeutic option.

Thus, it was the purpose of the present study to establish an intra-operative filtration model for disseminated tumour cells in patients who were undergoing liver surgery for metastases. In order to study the maximum number of intra-operative disseminated cells we connected the filtration system to a selective catheter within a major liver vein located close to the tumour.

## Methods

*Study design.* In an experimental study in consecutive patients who were undergoing liver surgery due to colorectal metastasis bone marrow (BM) samples were analysed to describe the dissemination of CK+ cells before surgery as a status of the metastatic potential of the primary tumour. Blood samples were taken from the hepatic veins located close to the tumour tissue, the suspected focus for the intra-operative cell release. Blood samples taken before surgery allow the detection of already circulating tumour cells; blood samples taken during hepatic mobilisation, therefore, indicate the influence of the surgical technique. The content of CK+ cells detected in blood before filtration was compared with the content of CK+ cells detected in the blood sample directly after passing the leukocyte adhesion filter WBF2 (Pall, USA). Owing to the sample volume of 500 ml for the combined blood-collection and in-line filtration system, citrate–phosphate–dextrose–adenine (CPDA) blood was re-transfused after it had passed through the filter.

*Patients.* The study was performed in 18 patients (ten male, eight female) with a median age of 60 years (range 43–74 years) who were undergoing liver surgery for hepatic metastasis of colorectal cancer (primary tumour stage UICC I–IV) at the Department of General and Transplantation Surgery at the University of Essen from March 1999 to March 2002. Patients were included in the study after they had given their written informed consent, which was obtained from all patients for the blood sampling and autotransfusion procedure and from 15 of the 18 patients for additional bone marrow aspiration. The study was performed according to the guidelines of the German law and approved by the local ethics committee. Patients were followed for survival and recurrent diseases for at least 2 years after surgery.

*Catheter placement.* After intubation and establishment of general anaesthesia the right jugular vein was punctured. A sidewinder angiography catheter (Ducor 7F-HighFlow MPA2, Cordis, Germany) was guided (Emerald guidewire, 150 cm, Cordis) under ultrasound control into one of the three major hepatic veins, which was located close the tumour; final catheter location was documented by X-ray.

*Bone marrow sampling.* Bone marrow samples (10 ml) were obtained after induction of general anaesthesia by a standard needle aspiration technique from both iliac crests (before laparotomy).

*Operation.* Liver resections were performed by standard surgical procedures, including hepatic mobilisation and control of major vessels (in the liver hilum and liver veins) prior to resection by the same surgical team. Manipulation of the tumour tissue was prevented as best as possible. The anterior approach for liver resection was not considered [9, 10].

*Blood sampling and filtration.* Directly after catheter placement and prior to laparotomy 27 ml of liver venous blood was sampled (preoperative sample). Starting at the beginning of the hepatic mobilisation another 500 ml liver venous blood were collected continuously within 30 min into the transfusion system (Leukotrap WB, Pall). After appropriate merging, an aliquot of 27 ml was taken from the 500 ml for later analysis (intra-operative sample).

To determine the efficacy of potential cell removal of the integrated filter medium we took another aliquot of 27 ml blood, after in-line filtration (WBF2 filter, Pall) but prior to re-transfusion, and analysed it for the content of CK+ cells (after-filtration sample).

*Preparation of bone marrow and blood.* Mononuclear cells (MNCs) were isolated from the heparinised bone marrow (5,000 U heparin/ml) and blood samples taken pre-operatively, intra-operatively, and after filtration (EDTA-containing monovettes) by Ficoll–Hypaque density-gradient centrifugation (density 1.077 g/ml; Pharmacia, Germany) at 400 *g* for 30 min. Interface cells were washed (400 *g* for 15 min) and resuspended in PBS. A total of $6–8\times10^6$ cells ($1\times10^6$ per slide) of each BM aspiration and $4–6\times10^6$ cells ($1\times10^6$ per slide) of each blood sample were spun directly onto glass slides (400 *g* for 5 min) coated with poly-L-lysine (Sigma, Germany) with a Hettich centrifuge (Mikro 22R, Hettich, Germany) [11].

*Immunochemistry.* After the cells had been air-dried overnight, staining for CK+ cells was performed with the Epimet kit (Micromet, Germany), based on the reactivity of the murine monoclonal antibody (Mab) A45-B/B3, directed against a common epitope of CK polypeptides [12]. The applied antibody A45-B/B3 for the detection of CK+ cells was shown to give no false-positive results as determined in a BM analysis of 165 patients without malignant diseases and is now commonly used [13]. The kit uses Fab fragments of the pan-Mab complexed with alkaline phosphatase molecules. The method includes permeabilisation of the cells with a detergent (5 min); fixation with a formaldehyde-based solution (10 min); binding of the conjugate Mab A45-B/B3-alkaline phosphatase to cytoskeleton CKs (45 min); and formation of an insoluble, red, reaction product at the side of binding of the specific conjugate (15 min). Subsequently, the cells were counterstained with Mayer's haematoxylin for 1 min and finally mounted with aqueous permanent mounting medium containing 15 mmol $NaN_3$ (Dako, Germany).

A negative control (antibody, conjugate of Fab fragment; Micromet) was served for each patient. A positive control with the colon carcinoma cell line HT-29 was stained under the same con-

ditions for each test. Microscopic determination was carried out independently by two investigators.

*Examination of data and statistics.* Patients were assessed as tumour cell positive if at least one CK+ cell was detected by immunochemistry. Patients' follow-up for survival and tumour recurrence was performed within 3-month intervals. Statistical analysis was done with the SPSS software package (version 11.0.0., SPSS, USA). Tumour cell detection in blood and bone marrow samples was compared with time-to-disease recurrence by the log-rank test and displayed in Kaplan Meier–curves. Sample correlation to standard prognostic criteria after resection of liver metastases (number and size of liver metastases and UICC stage of the primary tumour) was done by Fisher's exact test. $P$ values $\leq 0.05$ were counted as significant.

## Results

*Surgical procedure.* In seven patients a right hepatectomy (including three extended resections) was performed, in eight patients a left hepatectomy (including two left lateral, three extended resection); in three patients atypical liver resections were performed. Fifteen of 18 patients underwent major liver surgical procedures, defined as resection of three or more liver segments.

Mean operation time was 185 min (range 75–320 min). After laparotomy, the time range between liver mobilisation and the beginning of tumour resection was comparable in all cases. In five of 18 patients a Pringle manoeuvre was performed; mean time of partial vascular occlusion was 34 min; total surgical blood loss was less than 1,500 ml in all cases. Intra-operative courses were uneventful. In all patients a potential curative R0 resection could be achieved; post-operative histology of the resected specimen confirmed the presence of metastases of colorectal carcinoma in all cases.

*Catheter placement, blood collection and auto-transfusion.* Catheter placement was uneventful in all cases. In ten of 18 patients the catheter was placed in the right hepatic vein, in eight of 18 patients it was placed in the left hepatic vein. Sampling of 500 ml blood during mobilisation and resection via the catheter was done in all cases within 30 min, with no influence on the patients' haemodynamic stability. Auto-transfusion of CPDA blood after in-line filtration with the Leukotrap WB system was uneventful in all cases; haemolytic, thrombotic, embolic, or allergic side effects were not observed. Total blood loss due to sampling was less than 90 ml in all patients.

*Filtration.* Filtration of CPDA blood was by gravity, into a satellite bag of the blood collection system. CK+ cells were detected in 12 of 18 patients before in-line filtration. After passage through the filter, CK+ cells were detected only in one case. The adhesion of CK+ cells to the fibres of the filter medium could be demonstrated by light microscopy after immunochemistry. After retrograde perfusion of the filter medium with a saline solution a partial fraction of CK+ cells could be liberated from the filter medium. Figure 1 shows the reduction of CK+ cells in 11 of 12 CK+ patients after filtration with the WBF2 system.

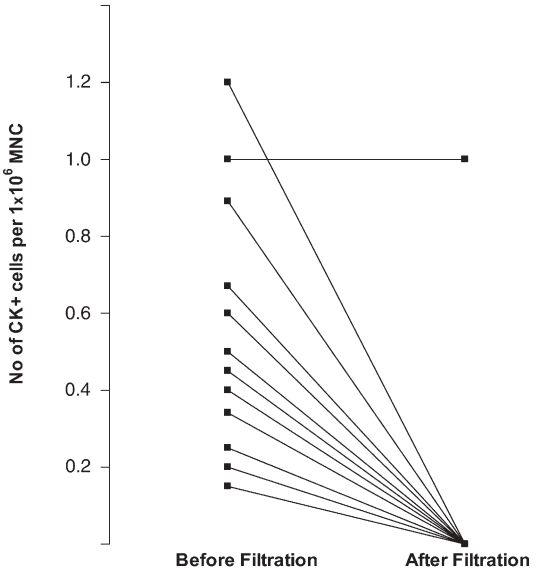


**Fig. 1** Effect of filtration; CK+ cells before and after filtration in 12 patients. *The squares* indicate the number of CK+ cells per $1\times10^6$ MNCs. Values <1 indicate the number of CK+ cells found in $6\times10^6$ cells totally analysed

*Distribution of CK+ cells.* BM analysis failed in one patient due to sample clotting. The applied antibody for the detection of CK+ cells was shown to give no false-negative results (Table 1).

CK+ cells were detected in BM of nine of 14 (64%) patients and in blood samples (at least one CK+ cell at any time point, pre-operatively and intra-operatively) of 12 of 18 (67%) patients. In seven of 14 (50%) patients CK+ cells were detected in BM and in blood samples. In two of 14 (14%) patients CK+ cells were detected only in BM, and in two of 14 (14%) patients CK+ cells were found in blood samples solely. There were no CK+ cells in three of 14 (21%) patients at any time. In ten of 18 (55%) patients CK+ cells were detected in the blood samples taken during hepatic mobilisation; in three of 14 (21%) patients tumour cells were detected only during surgery, possibly indicating intra-operative tumour cell dissemination; all these patients underwent major liver resection. The distribution of CK+ cells in BM and different blood samples, sorted by the initial UICC stage of the patients, is shown in Table 2. CK+ blood samples were found more often in patients with CK+ BM samples than in patients with CK– BM status (78% vs 22%).

**Table 1** Distribution of CK+ or CK– cells in BM and blood samples [pre-operative, during hepatic mobilisation (intra-operative), and after passage through the filter] in 18 patients. (*ND* not done, *E* sampling error)

| Patient no. | BM CK cells | Pre-operative blood CK cells | Intra-operative blood CK cells | Blood after filter CK cells |
|---|---|---|---|---|
| 1 | ND | – | – | – |
| 2 | ND | – | + | – |
| 3 | ND | – | + | – |
| 4 | E | + | – | – |
| 5 | – | – | – | – |
| 6 | – | – | + | – |
| 7 | – | – | – | – |
| 8 | – | – | – | – |
| 9 | – | + | + | – |
| 10 | + | – | – | – |
| 11 | + | + | + | – |
| 12 | + | + | + | – |
| 13 | + | – | – | – |
| 14 | + | – | + | – |
| 15 | + | + | + | + |
| 16 | + | – | + | – |
| 17 | + | + | + | – |
| 18 | + | + | – | – |

**Table 2** Detection of CK+ cells in BM and blood samples (total: at least one CK+ cell at any time point, pre-operatively and intra-operatively) in 14 of 18 patients, according to UICC status

| UICC (*n*=14) | BM CK+ | Blood CK+ | | |
|---|---|---|---|---|
| | | Total | Pre-operative | Intra-operative |
| I+II (*n*=4) | 3 (75%) | 2 (50%) | 2 (50%) | 1 (25%) |
| III (*n*=5) | 3 (60%) | 3 (60%) | 3 (60%) | 3 (60%) |
| IV (*n*=5) | 3 (60%) | 4 (80%) | 2 (40%) | 4 (80%) |

*Patient follow-up.* The mean follow-up time for all 18 patients was 31 months. Recurrent disease (RD) was observed in eight patients after a mean of 14 months. To date seven patients have died, within a mean of 20 months after surgery (range 12–45 months), with evidence of tumour recurrence. All four patients with UICC I and II are alive, one with known tumour recurrence (Fig. 2). The UICC status (according to the first diagnosis of colorectal cancer) and the number of metastases showed no significant statistic correlation with the distribution of CK+ cells (Fisher's exact test $P>0.05$).

CK+ cell detection in BM samples did not statistically correlate with RD (log rank $P=0.8856$). Whereas RD showed no significant correlation with the detection of CK+ cells in pre-operative blood samples (log rank $P=0.1194$), it was more often seen in patients with detection of CK+ cells in the intra-operative blood samples than patients with intra-operative negative findings (log rank $P=0.0162$).

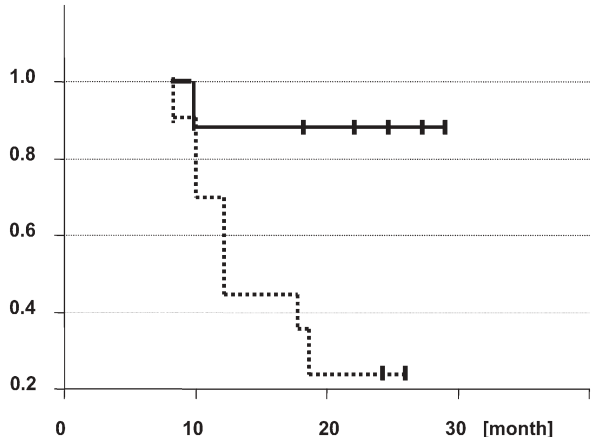


**Fig. 2** Kaplan–Meier survival analysis for time-to-disease recurrence in patients with (*dotted line*) or without (*solid line*) intra-operative detection of CK+ cells (log-rank $P=0.0162$)

## Discussion

The liver is a common site for colorectal cancer metastases. Owing to evolving surgical techniques most patients with hepatic metastases can be treated with curative intention [2]; untreated liver metastases results in a 5-year survival rate of less than 5% [14]. However, even after multimodal—potentially curative—treatment, up to 50% of patients will develop tumour recurrence. Thus, the main reason may be the spread of occult tumour cells. It has recently been demonstrated that mechanical alteration of the liver during resection of liver metastases may lead to massive intra-operative tumour cell shedding into the blood circulation [3]. Similar phenomena had been described before for colorectal cancer surgery, which resulted in concepts such as "no-touch" surgery [1, 15].

Although some patterns of pre-operative and intra-operative tumour cell dissemination in correlation with surgical manipulation have been described, little is known about the optimal place and time to detect these cells. In our opinion the study of tumour cell dissemination into the blood can be more effective by catheterisation of selected vessels. Koch et al. were able to show, in a comparative analysis of tumour cell dissemination in different blood compartments, that the detection rate of disseminated tumour cells was significantly higher in mesenteric venous blood than that in central and peripheral blood taken in patients who were undergoing resection of primary colorectal cancer [16]. This emphasises the importance of the location of sampling to detect early systemic haematogenic tumour cell dissemination.

Compared with all other reported studies in patients who had undergone liver surgery, blood sampling in our study focuses on the detection of cell release via the hepatic veins, before entering systemic circulation. The technique of liver vein catheterisation was first used in experimental studies that measured hepatic venous oxy-

gen saturation during surgery. Kainuma et al. inserted fibre-optic pulmonary artery catheters via puncture into the hepatic veins of 33 consecutive patients who were undergoing hepatic resection with a success rate of 100% and mean application time of 14 min [7]. Our results show that the use of this method for blood sampling via the catheter is safe and feasible for all patients; in only two patients non-sustained atrial arrhythmias were observed.

The best time and place for tumour cell detection can hardly be defined. Continuous blood sampling from the outflow of the liver veins during up to 30 min of hepatic mobilisation and a cumulative sample volume of 500 ml resulted, in our study, in an increased detection rate of CK+ cells (55%), compared to the results of Weitz et al. [3] of at least 15%. The cumulative incidence of CK+ cell detection during surgery was even higher than that in 34 patients with sample volumes of 27 ml, via a liver venous catheter, in a pilot series that had been performed in our department previously. In our patients major resection attempts and total number of metastases correlate with the intra-operative release of CK+ cells; in 21% of those patients CK+ cells were detected during mobilisation solely, indicating the absolute influence of surgery.

The rate of 64% CK+ cells detected in BM in our study is higher than in most other studies; Schlimok et al. found 26.9% CK+ cells in $1.5\times10^5$ BM cells [17] and Lindemann et al. detected 31.9% CK+ cells in $3\times10^5$ cells [18]. This may be explained by the higher number of analysed cells per patient (more than $1\times10^7$ cells) or by the patients' characteristics. Seven of nine patients with CK+ cells within BM had an initial UICC stage of III or IV.

In view of the fact that some filter media for leukocyte adhesion are able to remove tumour cells under in vitro conditions [5, 6], we sampled intra-operative blood into a third-generation filter-containing auto-transfusion system [19]. Using a filter with a cell adhesion-promoting polymer coating with polyethyleneimene-modified polyurethane films, we could demonstrate a reproducible, significant, depletion of CK+ cells up to 100% in vivo; in only one case CK+ cells were detected after the filtration procedure, possibly due to saturation kinetics of the filter medium. Although lower numbers of cells were available after filtration than before filtration, our applied filter system seems to be effective in retaining CK+ cells spread during surgery.

In animal studies the detection of circulating cell clusters was found to be a more important prognostic factor than the detection of single circulating cells [20]. Although most mechanisms of cell depletion still remain unclear [21], filtration technology has to be discussed as a therapeutic tool. In this finding, cell filtration can be a very effective therapy for disseminated cells, as the cells appear in the blood as tumour-cell clusters or mixed-cell clusters [22], for example, combined with the use of bypass filtration [8].

However, in our opinion the mobilisation phase of the liver carries out the highest risk for tumour cell dissemination during surgery, and surgical techniques to avoid this are rare. During hepatic resection, complete mobilisation of the right lobe of the liver, with the right hepatic vein controlled outside the liver before parenchymal dissection, has been advised by most surgeons. This "conventional approach" is helpful in reducing the amount of surgical blood loss. The anterior liver resection technique is one of the "non-conventional approaches" to advanced liver cancer in an attempt to avoid prolonged manipulation of the hepatic lobes, causing impairment of the afferent and efferent circulation. The technique involves initial completion of parenchymal dissection before the right lobe is mobilised. After control of the inflow vessels within the hilum, liver parenchyma is transected, e.g. by the use of an ultrasonic dissector, until the anterior surface of the inferior vena cava is exposed. The right hepatic lobe is then mobilised laterally by the securing of all venous tributaries, including the right hepatic vein [10, 23].

To date, the theoretic advantage of the anterior over the conventional approach has not been documented for the effects of tumour cell dissemination. In the present study the conventional approach was preferred, due to better standardisation and the potential dangers, as torrential bleeding can occur at the deeper plane of parenchymal dissection. In the future, the anterior approach might be one technique, if adequate experience exists. We have already shown with our animal model [8] that the combination of an extracorporeal bypass circuit and the conventional resection technique is feasible. Besides its use within bypass systems, the filtration method might also be of clinical interest in cell saver application during surgery.

As the detection of CK+ cells during surgery has to be discussed as an independent prognostic parameter, the results presented might help surgeons to think about modifying resection techniques to avoid or reduce the spreading of intra-operative tumour cells.

Based on these findings, we have already established clinical filtration models for patients who are undergoing liver surgery. As in ex situ resection techniques [24, 25] the patients underwent liver resection after veno–venous bypass with integrated leukocyte reduction filters within the circuit. Further studies will include the characterisation of CK+ cell spread during surgery, with respect to surgical technique, compared to those cells already present in the bone marrow before surgery. This might help the surgeon to identify patients with an increased risk of tumour recurrence who may benefit from adjuvant therapeutic strategies.

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