Annals of Surgical Oncology, **6**(7):658–663
Published by Lippincott Williams & Wilkins

Isolated Organ Perfusion Does Not Result in Systemic Microembolization of Tumor Cells

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Background: Isolated organ perfusion with hyperthermia and melphalan with or without tumor necrosis factor-α has been effectively used to treat regionally confined, unresectable malignancies of both the limb and liver. Many patients, however, will eventually relapse at distant sites. We used reverse transcription-polymerase chain reaction (RT-PCR) to determine whether significant tumor microembolization occurs in patients undergoing isolated limb perfusion (ILP), isolated hepatic perfusion (IHP), or hepatic resection.

Methods: Primers specific for the human tyrosinase gene or carcinoembryonic antigen gene were designed for RT-PCR to screen melanoma or colon adenocarcinoma, respectively. RNA from human melanoma lines (Pmel and 1286) and human colon adenocarcinoma lines (H508 and HT29) were used to generate positive control cDNA. Normal human blood was inoculated with tumor cells at concentrations that ranged from 10^{-2} to 10^{5} tumor cells/ml of blood to define the sensitivity. Systemic and perfusate blood samples were drawn from 15 patients (8 patients underwent IHP, 5 patients underwent ILP, and 2 patients underwent resection) before the start of the operation, immediately before and during the perfusion, and postoperatively. Mononuclear cell fractions were separated from the blood samples and RNA was extracted for the RT-PCR assay. Standard primers for human β-actin were used to confirm that cDNA was generated after the RT reaction.

Results: RT-PCR assay sensitivity was determined to be 10 tumor cells/ml of whole blood. Of the 8 IHP patients, 6 had colon metastases and 2 had ocular melanoma metastases to the liver. All 5 ILP patients had in transit melanoma of the extremity. Two patients with colon metastases to the liver were found to have resectable disease. There were no detectable circulating tumor cells in the systemic circulation either preoperatively or postoperatively in all 15 patients that were screened.

Conclusions: RT-PCR is a highly sensitive method of detecting tumor cells in perfusate or blood. Manipulation of the limb or liver followed by resection or isolated hyperthermic perfusion does not cause detectable release of circulating tumor cells. The late development of distant metastases observed in many of these patients does not correlate with the ability to measure circulating tumor cells during regional therapy.

Key Words: Isolated organ perfusion—RT-PCR—Carcinoembryonic antigen—Tyrosinase—Tumor microembolization.

Vascular isolation and perfusion of the limb or liver by using melphalan with or without tumor necrosis factor can result in high response rates for patients with intransit extremity melanoma,[1,2] unresectable high-grade extremity sarcoma,[3,4] and unresectable primary metastatic cancers confined to the liver.[5,6] Despite the frequent complete or partial regression of tumors within the perfusion field, eventual progression of tumor at systemic sites occurs in at least half of the patients.[6,7] Although patients with regionally advanced cancers are at risk for systemic disease, it is possible that during vascular isolation and perfusion of an extremity or the liver, there are factors related to treatment that may promote significant microembolization of tumor into the systemic circulation. Factors related to the perfusion itself, such as nonphysiological flow dynamics or

Received March 6, 1999; accepted July 12, 1999.

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Presented at the Society of Surgical Oncology Annual Meeting, Orlando, FL, March 4–7, 1999.

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changes in pH, may result in embolization of tumor cells via unknown mechanisms. In a similar manner, it is possible that melphalan, tumor necrosis factor, or associated inflammatory or vasoactive effects that these agents have on the tumor microvasculature may cause release of viable tumor emboli during reperfusion of the extremity immediately after treatment.

Reverse transcription-polymerase chain reaction (RT-PCR) has been shown to be a sensitive method of detecting small numbers of tumor cells in peripheral blood, lymph nodes, or bone marrow in patients with melanoma,[8–11] colorectal cancer, or breast cancer.[12–16] Although the significance of circulating tumor cells identified by RT-PCR in patients has not been clearly defined,[11,14,17] the technique has been established as a sensitive method of detecting between 1 and 10 cells/ml of blood.[8,16] Because of the high frequency of systemic metastases that occurs in patients after isolated organ perfusion of the limb or liver, the current study was undertaken to determine whether or not treatment with isolated organ perfusion results in release of tumor cells into the perfusion circuit or systemic circulation during or soon after treatment.

MATERIALS AND METHODS

Patient Population

Between January 1997 and January 1998, 15 patients were studied and followed prospectively, which included 8 patients who had undergone isolated hepatic perfusion (IHP), 5 patients who had undergone isolated limb perfusion (ILP), and 2 patients who had undergone hepatic resection. All patients were enrolled in Surgery Branch treatment protocols approved by the Institutional Review Board and the Cancer Therapeutics Evaluation Program of the National Cancer Institute. Patients with regionally confined, unresectable malignancies of the liver were treated with a 60-minute hyperthermic IHP, using 1.5 mg/kg of melphalan (Glaxo Wellcome, Research Triangle Park, NC) with or without 1.0 mg tumor necrosis factor (Knoll Pharmaceuticals, Whippany, NJ). The technique of IHP was performed as previously described.[6] Of the 8 patients who underwent IHP, 6 had isolated colon metastases to the liver and 2 had isolated ocular melanoma metastases to the liver. Two patients with isolated hepatic metastases from colon adenocarcinoma were found to have resectable disease and underwent hepatic resection. All 5 patients who underwent ILP had in transit melanoma of the extremity and were treated with a 90-minute hyperthermic ILP, using 10 mg of melphalan/L of limb volume with or without 4.0 mg of tumor necrosis factor as previously described.[18]

Patient Samples

Systemic and perfusate blood samples were drawn from each patient undergoing either IHP or ILP as follows: (1) preoperative systemic blood, (2) systemic and perfusate blood at the start of perfusion, (3) systemic and perfusate blood at the conclusion of perfusion, and (4) systemic blood 2 hours after perfusion. In the two patients who underwent hepatic resection, preoperative and postoperative systemic blood samples were collected. Each sample consisted of 8 ml of blood placed into Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ) for separation of mononuclear cells from whole blood by using a Ficoll Hypaque density fluid and a polyester gel barrier. Sample tubes were centrifuged at room temperature for 20 minutes at 1800 relative centrifugal force. The resultant mononuclear cell band was collected, washed twice in sterile phosphate-buffered saline (Biofluids, Rockville, MD), and pelleted by centrifugation. Total RNA was extracted from both cultured cell lines and patient samples by using Rneasy Total RNA kits (Qiagen, Chatsworth, CA) according to the manufacturer's protocol.

Tumor Cell Lines

Pmel and 1286 are primary human melanoma lines derived from patients treated at the National Cancer Institute. H508 and HT29 are colon adenocarcinoma lines obtained from the American Type Culture Collection (Rockville, MD). All tumor cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin/streptomycin (Biofluids) at 37°C in a 5% CO_2 incubator. Each of the lines was passaged for no more than eight generations and cryopreserved at regular intervals.

Tumor Immunohistochemistry

Hematoxylin and eosin slides of the patients' surgical material were reviewed and appropriate sections that contained both tumor and normal tissue were selected for staining with carcinoembryonic antigen (CEA) or S-100. Paraffin-embedded sections were stained for CEA or S-100 by standard immunohistochemical techniques, using a Ventana automated immunohistochemistry stainer (Ventana Medical Systems, Tucson, AZ). The sections were stained with a monoclonal antibody for CEA or S-100. Two patients with ocular melanoma to the liver had heavy pigmentation in the tumor on pathological examination. Slides were reviewed and scored as positive if the staining had a membranous and cytoplasmic pattern in the tumor cells, distinctly stronger than the background staining of the surrounding normal tissue.

Primers

The following oligonucleotide primers were designed from GenBank sequences of human CEA and tyrosinase (TYR) and synthesized (Bio-Synthesis, Inc., Lewisville, TX) for PCR amplification, as follows: CEA sense 5′-GCGCAGTGATTCAGTCATCC-3′, CEA antisense 5′-GCAGGAGAGGCTGAGGTTCA-3′, TYR sense 5′-TTGGCAGATTGTCTGTAGCC-3′, and TYR antisense 5′-GCTATCCCAGTAAGTGGACT-3′. CEA primers generated a PCR product of 699 base pair (bp). TYR primers amplified a PCR product of 254 bp.

Primers for human β-actin were also designed, resulting in a 660-bp product that was used to confirm RNA integrity for RT-PCR as follows: β-Actin sense 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, and β-actin antisense 5′-CTAGAAGCATTTGCGGTG-GACGATGGAGGG-3′.

RT-PCR Method

Extracted total RNA (1 μg) was incubated with 1.5 μl oligo(dT)$_{12–18}$ primer (0.5 mg/ml; Life Technologies, Inc., Rockville, MD) and diluted with diethyl polycarbonate-treated distilled water to a volume of 33 μl at 70°C for 10 minutes and quick-chilled on ice. To this mixture was added 12 μl of 5 × first strand buffer (Life Technologies), 6 μl of 0.1 M dithiothreitol (Life Technologies), and 6 μl of 10 mM dNTP (Life Technologies), and incubated at 40°C for 5 minutes. To produce cDNA, 3 μl of Moloney murine leukemia virus reverse transcriptase (Life Technologies) was added and incubated at 40°C for 1 hour and chilled on ice.

PCR was then performed on the cDNA templates by using PCR SuperMix (Life Technologies) containing 22 mM Tris-HCl, 55 mM KCl, 1.65 mM $MgCl_2$, 220 μM dGTP, 220 μM dATP, 220 μM dTTP, 200 μM dCTP, and 22 U recombinant *Taq* DNA polymerase/ml in a total reaction volume of 55 μl with 200 nM of each primer under the following conditions: one cycle of 5 minutes at 92°C for template denaturation, followed by 25 cycles of 15 seconds' denaturation at 94°C, 30 seconds at 55°C for primer annealing, and 2 minutes at 72°C for polymerase extension. All PCR reactions were terminated with a 7-minute extension at 72°C. Final reaction products were electrophoresed on 2% agarose gels and visualized on an ultraviolet transilluminator after ethidium bromide staining. All gels were analyzed with a 100-bp DNA ladder (Life Technologies). Water negative controls contained all components of the RT-PCR reaction, but no target RNA.

RNA integrity for RT-PCR assay was determined by always performing parallel reactions using primers for β-actin, producing a fragment of 660 bp. Samples that failed to amplify the β-actin product were considered noninformative.

Sensitivity Testing

Tumor cell inoculation experiments were performed to determine the sensitivity of the RT-PCR assay to detect tumor cells suspended in whole blood. Known concentrations of H508 colon adenocarcinoma cells were added to donor whole blood, total RNA extracted, and RT-PCR performed for detection of CEA as described above. Serial dilutions were performed to cover a range of 10^5 to 10^{-2} tumor cells/ml. A normal blood sample was included in each experiment. Normal whole blood contains between 1×10^6 and 1×10^7 white mononuclear cells/ml. These experiments were repeated in triplicate.

RESULTS

Figure 1 shows the primer controls for detecting human CEA and TYR in the colon carcinoma lines H508 and HT29, and human melanoma lines Pmel and 1286, respectively. The CEA primers detected a fragment of 699 bp from both colon carcinoma lines. The TYR primers amplified a 254-bp fragment from the human melanoma cell lines. The quality of isolated RNA was confirmed by parallel amplification with β-actin primers for each RT-PCR reaction as evidenced by the 660-bp fragment on gel electrophoresis.

H508 colon carcinoma cell inoculation experiments in donor blood demonstrated that RT-PCR assay sensitivity was 10 tumor cells/ml of whole blood as shown in Fig. 2. There were no RT-PCR products detected in whole blood alone. The band intensity increased with increasing concentrations of tumor cells. In one of three experiments, a 699-bp RT-PCR product was seen at 1 tumor cell/ml.

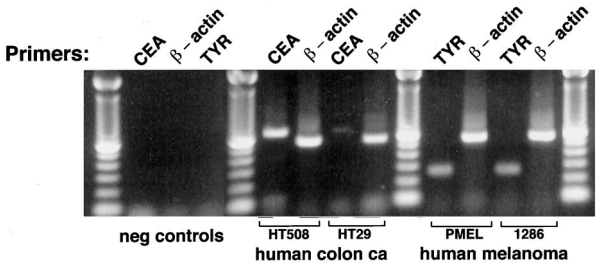

FIG. 1. Reverse transcription-polymerase chain reaction (RT-PCR) assay controls. Carcinoembryonic antigen (CEA) primers detect a 699-bp fragment in both human colon adenocarcinoma lines tested (H508 and HT29). Tyrosinase (TYR) primers amplify a 254-bp fragment from the human melanoma lines Pmel and 1286. β-Actin primers produce a 660-bp fragment to ensure that RNA was of sufficient purity for RT-PCR. Negative water controls are shown.

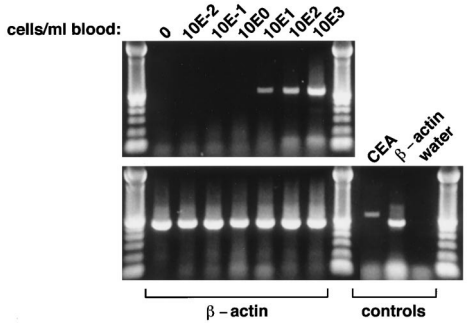

FIG. 2. Assay sensitivity. Serial dilutions of donor blood spiked with H508 colon carcinoma cells. The upper gel shows the assay sensitivity to detect the 699-bp fragment, using carcinoembryonic antigen (CEA) primers. The lower gel shows consistent amplification of β-actin in all samples including naive whole blood in the first sample lane. Controls are shown in the lower right.

Table 1 summarizes 15 patients according to tumor histology, site of disease, and treatment received. In addition, preoperative serum CEA levels are shown. All patient tumor specimens, with one exception, were tested for appropriate tumor marker expression by immunocytochemistry. One patient with isolated colon metastases to the liver could not be tested, but it is noted that this patient had a serum CEA level of 3900 ng/ml. All patients with melanoma had either pigmented tumors or tumors that stained positive for S-100 or both. Follow-up information including sites of recurrent disease and distant metastases after treatment are shown.

RT-PCR analysis of preoperative, intraoperative, and postoperative blood specimens from all 15 patients failed to demonstrate the presence of circulating tumor cells by screening for CEA or TYR in patients with colon carcinoma or melanoma, respectively, despite amplification of appropriate controls, using matching primer pairs. Figure 3 shows representative data from six patients, two patients each undergoing IHP, hepatic resection, and ILP. Patients 1, 3, and 6 had no evidence of disease progression after treatment. In contrast, Patients 2, 4, and 5 developed both tumor progression and distant metastases within months after treatment. Patient 2 developed lung metastases after IHP for colon metastases to the liver. Patient 4 had disease recurrence in the liver after hepatic resection for metastatic colon carcinoma. Patient 5 developed brain metastases after ILP for in transit melanoma of the extremity. Despite a variety of patient outcomes after treatment, there was no observable correlation between detectable release of circulating tumor cells after regional therapy and the development of distant metastases.

Of interest, no β-actin band was detected in the perfusate samples obtained at the beginning of perfusion (lane e, Fig. 3). This is explained by the fact that the perfusate composition initially contains one unit of packed red blood cells and a balanced salt solution and no nucleated cells to provide an RT-PCR β-actin product. That a β-actin band was consistently present at the end of treatment indicates that nucleated host cells were released into the perfusate from liver during perfusion (lane f, Fig. 3).

DISCUSSION

The current study is the first to show that there does not appear to be release of circulating viable tumor cells

TABLE 1. *Patient summary and outcome*

Patient No.	Histology	Site of disease	Treatment	Preoperative CEA level (ng/ml)	Tumor expression	Follow-up status
1	Colon adenocarcinoma	Liver	IHP	41	CEA+	Liver/peritoneum/SQ
2	Colon adenocarcinoma	Liver	IHP	1793	CEA+	Liver
3	Colon adenocarcinoma	Liver	IHP	48	CEA+	Liver/lung
4	Colon adenocarcinoma	Liver	IHP	11	CEA+	Liver
5	Colon adenocarcinoma	Liver	IHP	1519	CEA+	Liver/lung
6	Colon adenocarcinoma	Liver	IHP	3900	NA	Stable
7	Colon adenocarcinoma	Liver	Hepatic resection	205	CEA+	NED
8	Colon adenocarcinoma	Liver	Hepatic resection	20	CEA+	Liver
9	Ocular melanoma	Liver	IHP	NA	Pigment+	Liver
10	Ocular melanoma	Liver	IHP	NA	Pigment+	Liver/lung
11	Melanoma	Lower extremity	ILP	NA	S-100+	NED
12	Melanoma	Lower extremity	ILP	NA	S-100+	NED
13	Melanoma	Lower extremity	ILP	NA	S-100+	NED
14	Melanoma	Lower extremity	ILP	NA	S-100+	Limb
15	Melanoma	Lower extremity	ILP	NA	S-100+	Brain

CEA, carcinoembryonic antigen; IHP, isolated hepatic perfusion; SQ, subcutaneum; NA, not available; NED, no evidence of disease; ILP, isolated limb perfusion.

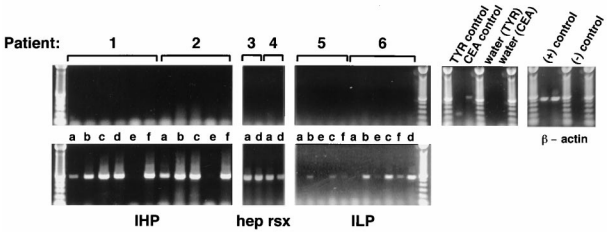

FIG. 3. Reverse transcription-polymerase chain reaction (RT-PCR) assay of patient samples for either carcinoembryonic antigen (CEA) or tyrosinase (TYR). The upper gels show RT-PCR results for amplification of tumor markers in either systemic or perfusate blood along with assay controls to the right. The samples are designated as follows: preoperative systemic blood (a); systemic blood at start of perfusion (b); systemic blood at end of perfusion (c); systemic blood 2 hours after perfusion (d); perfusate blood at the start of perfusion (e); and perfusate blood at the end of perfusion (f). The lower gel shows matching β-actin screening. IHP, isolated hepatic perfusion; hep rsx, hepatic resection; ILP, isolated limb perfusion.

secondary to isolated organ perfusion of the limb or liver and suggests that development of systemic disease after isolated organ perfusion is secondary to the progression of pre-existent micrometastatic disease. To establish the sensitivity of our experimental conditions, known quantities of tumor cells were added to normal blood in decreasing concentrations and then subjected to the experimental RT-PCR conditions used on perfusate and blood samples for patients undergoing treatment. Our data show that the primers are specific and consistently sensitive enough to detect 10 cells/ml of whole blood. In one experiment, sensitivity testing did reveal an RT-PCR product of appropriate size when as few as one cell was added to 1 ml of normal blood, consistent with previous reports.[8,19] Therefore, in fact, the conditions used may have been capable of detecting somewhere between 1 and 10 tumor cells/ml of blood. Five of seven patients treated with ocular or extremity melanoma had expression of S-100 on immunohistochemistry or had pigmented tumors. Seven of eight patients with colorectal cancer had CEA-expressing tumors. The one patient not tested for CEA expression by immunohistochemistry had a circulating preoperative CEA level of almost 4000 ng/ml.

Of the 10 patients who had isolated hepatic metastasis to the liver, 2 underwent resection with curative intent. One remains free of disease, but the other developed a new lesion in the remaining lobe of the liver. Previous data have shown no detectable circulating melanoma cells, using RT-PCR perioperatively in uveal melanoma patients.[20] Of the 6 patients who underwent IHP, 3 developed systemic metastases; and of the 5 patients who underwent ILP for melanoma, 2 developed metastases after treatment. Despite the heterogeneous nature of the outcomes in the patients tested, no positive RT-PCR product for CEA or TYR was identified in any patient at any time point from blood or perfusate samples. This indicates that the likelihood of significant embolization of viable tumor cells during perfusion or immediately after in these patients is low or nonexistent. Previous studies have shown that patients with in transit melanoma or those with extensive unresectable metastases to the liver from colorectal cancer are at high risk for developing systemic disease even in the absence of being subjected to isolation perfusion.[7,21]

The use of RT-PCR to detect occult cancer cells in circulating blood or regional lymph nodes for a variety of histologies has been under critical evaluation over the past several years. By using RT-PCR for tumor-specific markers such as CEA,[12,17,22] TYR,[9–11,23] prostatic specific antigen,[15] and cytokeratin,[16] circulating tumor cells in peripheral blood of cancer patients have been identified. Nested PCR has been advocated as a more sensitive method of detecting circulating neoplastic cells in peripheral blood and is sensitive enough to detect 1 cell in 10^6 molecular cells.[24] On the other hand, the diagnostic specificity is decreased.[25] In the current study, single-round PCR was used on peripheral blood and on perfusate. For the perfusion circuit, which is a closed 1-liter recirculating system, single-round PCR is, most likely, sensitive enough to screen for significant emobolization of tumor cells. In addition, single-round PCR is likely to identify whether significant systemic tumor emobilization occurs after reperfusion.

One of the most interesting aspects of this study is the finding obtained from perfusate samples at the beginning and end of treatment. No β-actin RT-PCR product was obtained at the beginning of treatment in the perfusate, which is consistent with the fact that the perfusate composition initially consists of 1 unit of packed red blood cells, mixed with a balanced salt solution. Therefore, no nucleated blood cells should be present to provide a subsequent β-actin RT-PCR product. On the other hand, at the end of the perfusion, in every circumstance there was a strong RT-PCR β-actin band, consistent with the release of host nucleated whole cells from the treated organ into the perfusate. That no detectable RT-PCR CEA or TYR product was detectable indicates that there were significant numbers of nucleated host, but not malignant, cells released into the perfusion circuit. This finding may indicate most convincingly that malignant cells are not released into the circulation secondary to the effects of isolated organ perfusion. This preliminary report is the first to show lack of circulating tumor cells in perfusate or circulating blood immediately during or after isolation perfusion. These data, although very con-

sistent, warrant continued investigation with larger patient cohorts.

Acknowledgments: We thank Drs. Michael Nishimura (Bethesda, MD), Michael Gnant (Vienna, Austria), Steven Rosenberg (Bethesda, MD), and Adam Riker (Bethesda, MD) for helpful comments and suggestions.

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