Transient Induction of E-Selectin Expression Following TNFα-Based Isolated Limb Perfusion in Melanoma and Sarcoma Patients Is Not Tumor Specific

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Summary: Endothelial injury of the tumor microvasculature after isolated limb perfusion (ILP) with TNF-α and melphalan is considered to play an important role in the pathogenesis of tumor necrosis. It is thought to follow endothelial cell activation and subsequent attraction of polymorphonuclear cells (PMNs). The observed selectivity for the tumor could be due to preferential overexpression of cell-adhesion molecules by the tumor vasculature. We tested this proposition by analyzing sequential biopsies from both tumor and normal distant skin, taken from melanoma and sarcoma patients before ILP and at 30 min and 24 h after ILP. Histopathologically confirmed complete response was observed in six of seven melanoma patients, 1–8 months after ILP. By using immunohistochemistry on the light- and electron-microscopic level, the expression patterns of intercellular adhesion molecules-1 (ICAM-1), E-selectin (ELAM-1), VCAM-1, and PECAM-1 were examined. In addition, the results were compared with the effects on HUVECs (human umbilical vein endothelial cells) in vitro of transient exposure of the agents used during ILP. ICAM-1 and PECAM-1 were constitutively expressed on vascular endothelial cells, both in normal tissues and in the tumor lesions. In biopsies taken 30 min after termination of the perfusion, a moderate induction of E-selectin expression on the vascular endothelium in the tumors and a marked expression on the vasculature in the perfused normal skin were observed. It decreased within 24 h after perfusion in both normal skin and in the tumor. The upregulation of E-selectin was accompanied neither by an influx of neutrophils nor by hemorrhagic necrosis. There were no drastic changes in the expression of VCAM-1, ICAM-1, or PECAM-1. These findings imply that the upregulation of E-selectin after ILP is not restricted to the tumor microvasculature and that, therefore, these microvascular events seem not to be the decisive pathomechanism responsible for tumor regression. Key Words: Melanoma—Sarcoma—TNF-α—Melphalan—Isolated limb perfusion.
of TNF-α in a closed compartment with acceptable side effects. The protocol used in our center involved a triple-drug regimen, based on the reported synergism of TNF-α with melphalan and interferon-γ (IFN-γ) supplemented with mild hyperthermia. In melanoma-in-transit metastasis (stage IIIA,B) a 90% complete response (CR) was obtained compared with ~40% CR rate after ILP with melphalan alone (5), a regimen cited usually in the literature. In inresectable soft-tissue sarcoma, this protocol was found to yield 50% CR with 87.5% limb salvage, because most tumors became resectable (6.7). The Department of Surgery, Dr. Daniël den Hoed Cancer Canter, Rotterdam, The Netherlands, participated in a clinical trial to compare this triple-drug regimen with a double-drug regimen with TNF-α and melphalan. The double-drug treatment was used in 16 of 17 patients analyzed in this study.

Angiographic analysis performed 1 week to 10 days after ILP showed a dramatic diminishment of the tumor-associated vasculature, leaving the normal small vessels in the limb intact, including small vessels in close proximity to the tumor (8). Morphologic and immunohistochemical analysis of biopsies of melanoma and soft-tissue sarcoma lesions after ILP with the triple-drug regimen suggested that the tumor microvasculature is an important target for ILP. We and others showed previously that events like Von Willebrand factor (VWF) release, platelet aggregation, and congestion concentrated on the tumor vasculature, leaving the normal tissues largely unaffected (9,10). Moreover, Renard et al. (9) observed E-selectin and VCAM-1 expressed by the endothelium followed by a massive polymorphonuclear (PMN) accumulation in both sarcoma and melanoma lesions after ILP with the triple-drug regimen.

From these findings, the authors suggested a multistep process of tumor destruction after cytokine administration, starting with intratumoral endothelial cell perturbation. This is manifested by overexpression of cellular adhesion molecules and followed by activation of PMNs, endothelial injury, and finally coagulative and hemorrhagic necrosis. A similar process has been described in experimental models (11). In accordance with this model, the observed specificity of the treatment for tumor tissue is a specific upregulation of adhesion molecules in the tumor vascular bed. In the non-involved tissues, the expression should be largely unaltered. However, a comparison with unexposed and exposed normal skin without tumor involvement was not made.

In our study, we challenged this proposed selectivity by analyzing sequential biopsies from both tumor lesions and normal distant skin from melanoma and sarcoma patients with extremity lesions. Biopsies were taken before ILP and 30 min and 24 h after termination of ILP with the double-drug regimen. We determined the expression patterns of the cellular adhesion molecules ICAM-1, E-selectin (ELAM-1), VCAM-1, and PECAM-1 (CD31) by immunohistochemical analysis on the light-microscopic (LM) and electron-microscopic (EM) level. In addition, the results were compared with in vitro (12) expression patterns on cultured huVECs (human umbilical vein endothelial cells) to be able to delineate possible synergistic effects of TNF-α and melphalan.

**PATIENTS AND METHODS**

**Patients and Treatment Schedule**

Specifications of the patient characteristics and biopsy samples are given in Table 1.

Seventeen patients who entered the ILP protocol in the Dr. Daniël den Hoed Cancer Canter, Rotterdam, The Netherlands, during the period between December 1993 and July 1994, were included in the study. Pertinent details about the inclusion criteria, types of drugs, and treatment schedule used were described earlier (5,6,8). ILP consisted on a 1.5-h perfusion with a total amount of 2–4 mg TNF-α in combination with melphalan, either 10 mg/L of perfused lower limb or 13 mg/L for the upper limb at mild hyperthermia (40°C). Sixteen patients were treated as follows. At time zero, TNF-α was injected as a bolus into the arterial line. Melphalan was administered 30 min later. One patient received 0.2 mg IFN-γ s.c. on 2 subsequent days before ILP. The ILP consisted of 0.2 mg IFN-γ, 4 mg TNF-α and melphalan, 10 mg/L in the perfused lower limb. In all patients, the limb was washed twice with 1 L of haemaccel and 1 L of 6% dextran 70 (Macrodex; Pharmacia, Uppsala, Sweden) at the end of ILP. Radioiodinated human serum albumin was added to the perfusate to monitor potential leakage of the drugs into the systemic circulation. Perioperatively, dopamine was administered at 2 μg/kg/min by continuous infusion, usually for several hours up to 48 h. Fluid loading was applied before releasing the tourniquet at the end of the washing period.
E-SELECTIN EXPRESSION

TABLE 1. Patient characteristics and specification of biopsy samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Normal skin (Number of biopsies)</th>
<th>Tumor (Number of biopsies)</th>
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<td>Before ILP 30 min after ILP 24 h after ILP</td>
<td>Before ILP 30 min after ILP 24 h after ILP</td>
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<tr>
<td>1st</td>
<td>Melanoma</td>
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<tr>
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<td>Melanoma</td>
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<tr>
<td>3rd</td>
<td>Melanoma</td>
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<tr>
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<tr>
<td>6th</td>
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<tr>
<td>7th</td>
<td>Melanoma</td>
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<tr>
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<td>Clear-cell sarcoma</td>
<td>1 1 1</td>
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<tr>
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<td>2 2 2</td>
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<tr>
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<td>Clear-cell sarcoma</td>
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<td>1 1 1</td>
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<td>Myxoid liposarcoma</td>
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<td>Synoviosarcoma</td>
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<td>17th</td>
<td>Lymphangiosarcoma</td>
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ILP, isolated limb perfusion.
* Material for immunoelectron microscopic analysis also was taken.

Oral informed consent to take sequential biopsies from normal skin of the involved extremity and the tumor lesions was obtained from the patients. Excision biopsy of normal skin was taken at least 15 cm outside the melanoma or sarcoma lesion in areas without macroscopic signs of disease. In total, 137 biopsies from 17 patients (excision and punch biopsies from melanoma patients and Trucut needle biopsies from sarcoma patients). From six patients samples from both normal skin and tumor tissue before, 30 min after, and 24 h after termination of ILP were taken and prepared for immunoelectron microscopy (IEM).

The responses to the ILP treatment—CR, partial response (PR), no change (NC), and progressive disease (PD)—were assessed by standardized criteria (13). In the melanoma patients, the response was histologically confirmed by a biopsy 1 to 8 months after perfusion. In sarcoma patients who underwent a resection or residual mass, extensive histologic examination of the resected specimen was performed. Six of seven melanoma patients had a CR to the treatment, whereas one had a PR. All sarcoma patients showed tumor softening and necrosis. One sarcoma patient had a CR with 100% necrosis, whereas nine of 10 had a PR.

Immunohistochemistry

Cryostat sections (4 μm) of normal skin, melanoma, and sarcoma lesions after ILP were air-dried overnight at room temperature (RT), fixed in acetone for 10 min, again air-dried and incubated with the primary antibody for 60 min. The following antibodies were used: PAL-E, recognizing endothelial cells (14,15) (our laboratory, undiluted supernatant); KH 58.01 POS, recognizing RaHuVWF (Von Willebrand factor; 1:400); CL203, recognizing ICAM-1 (16) (CD54 1:800; kindly provided by Dr. S. Ferrone); ENA-1, recognizing E-selectin (17), (CD62e 1:20; kindly provided by Dr. W. Buurman); 1G11, recognizing VCAM-1 (18) (CD106 1:20; Immunotech); BBA7, recognizing PECAM (19) (CD31 1:5000; British Biotechnology). Subsequently, sections were incubated for 30 min with peroxidase-labeled rabbit anti-mouse antibodies (Dako, 1:100) or with the ABC detection kit, according to the manufacturer's description (Vector, Burlingame, CA, U.S.A.). Each incubation was followed by extensive washing in phosphate-buffered saline (PBS). Peroxidase activity was visualized by incubation with the chromogen 3-aminon-9-ethylcarbazole for 10 min. Sections were counterstained with Harris hematoxylin and mounted with Kaiser's glycerin. The whole procedure was performed at RT. Secondary antibodies were tested for cross-reactivity by omitting the primary antibody, and positive controls were performed by staining frozen sections of skin affected with acute or chronic inflammation.

The staining intensity of the endothelium and tumor cells was scored as negative (−), weak (±),
for 1.5 h at RT, the sections were incubated in a 5
ammonium-nickel-sulfate-hexahydrate. The sections
were then developed for 1 min with
3 mg/ml diaminobenzidine (DAB; Sigma, St. Louis,
MO, U.S.A.) solution in PBS, containing 0.025% glutaraldehyde in PBS for 10 min at RT. The wells were incubated with 1% gelatin in PBS for 30 min at 37°C to prevent nonspecific binding of antibodies. After washing with PBS, the wells were successively incubated for 30 min at 37°C with the primary antibodies and with peroxidase-labeled rabbit anti-mouse immunoglobulin (diluted 1:500 in PBS, containing 1% gelatin). Enzyme substrate was then added (8 mg/ml 5-aminosalicylic acid; Sigma) in 50 mM phosphate buffer (pH 6.0) with 0.025% H₂O₂. Color development was measured after 30 min by reading the optical density (OD) at 450 nm in a Titertek ELISA plate reader (Flow Laboratories, Irvine, Scotland).

RESULTS

Immunohistochemical Analysis

The results in this section are presented per individual adhesion molecule; staining intensity is expressed as described in the Materials and Methods section, followed by the percentage of positively stained blood vessels (100%, all PAL-E-stained vessels).

PECAM-1 Expression

In frozen sections of normal skin before ILP, marked PECAM-1 expression was detectable on the endothelial cells of venules, arterioles, and large vessels (+ + +, 76–100%). The PAL-E–negative lymphatic endothelium also was positively stained with dull intensity (+). Melanoma and sarcoma cells did not stain. Vascular endothelial cells in either the melanoma or sarcoma lesions were marked E-selectin, ICAM-1, or VCAM-1 expression, as described previously (21). HUVECs were seeded in gelatin-coated 96-well microtiter plates (Costar, Cambridge, MA, U.S.A.) at a density of 2 × 10⁴ cells/well and allowed to grow to confluence in the appropriate medium for 3 days. Subsequently the cells were incubated with 10 μg/ml TNF-α (a gift from Boehringer Ingelheim) or melphalan, 40 μg/ml, or perfusate, obtained during ILP and containing the actually used dose of TNF-α and melphalan, for 1 h. After extensive washing, cells were incubated in the culture medium again. After various intervals, cells were washed with EMEM (Eagle’s minimal essential medium, Biowhittaker, Belgium) and fixed with 0.025% glutaraldehyde in PBS for 10 min at RT. The wells were incubated with 1% gelatin in PBS for 30 min at 37°C to prevent nonspecific binding of antibodies. After washing with PBS, the wells were successively incubated for 30 min at 37°C with the primary antibodies and with peroxidase-labeled rabbit anti-mouse immunoglobulin (diluted 1:500 in PBS, containing 1% gelatin). Enzyme substrate was then added (8 mg/ml 5-aminosalicylic acid; Sigma) in 50 mM phosphate buffer (pH 6.0) with 0.025% H₂O₂. Color development was measured after 30 min by reading the optical density (OD) at 450 nm in a Titertek ELISA plate reader (Flow Laboratories, Irvine, Scotland).

ELISA

An enzyme-linked immunosorbent assay (ELISA) on cultured HUVECs was performed to detect
not altered, both at 30 min and at 24 h after ILP (Figs. 1 and 3C). The melanoma and sarcoma cells remained unstained. IEM showed that the majority of the blood vessels were intensely labeled by the anti-PECAM-1 antibody at the luminal and abluminal side of the endothelium. Parts of the pinocytotic vesicles, mainly at the luminal side, were positively stained. No other part of the microvasculature showed PECAM-1 positivity (Fig. 3D).

ICAM-1 Expression

Before ILP, ICAM-1 was constitutively expressed in frozen sections of normal skin on vascular endothelial cells (+, 51-75%) and on dermal dendritic cells with dull staining intensity (+). The tumor vascular endothelium stained (+, 51-75%) in the melanoma and sarcoma lesions before ILP. In all melanoma lesions, tumor cells and stroma cells (+, 51-75%) were stained. Thirty minutes after termination of ILP, the staining intensity on the endothelial and dermal dendritic cells increased (from + to + +), both in normal skin and in the melanoma and sarcoma lesions. The percentage of the vessels stained remained unchanged (Fig. 5E). The staining of tumor cells and stromal cells remained unchanged. At 24 h after ILP, the staining intensity was decreased to the level observed before ILP (+, 51-75%), both in normal skin and in the tumor lesions (Figs. 1 and 3E). IEM analysis showed a marked ICAM-1 staining of the luminal side of endothelial cells. Sparingly pinocytotic vesicles were stained. No other part of the microvascular wall showed ICAM-1 staining (Fig. 3F).

E-Selectin Expression

In normal skin before ILP, the external sheath of the hair follicles markedly stained (+ + +), in contrast to the unstained vascular endothelial cells. Whether this staining could be considered specific or nonspecific was not known. Melanoma and sarcoma cells did not stain. The tumor vascular endothelium showed a weak staining (±, 5%) at the periphery of the tumor in two (of seven) melanomas and two (of 10) sarcoma lesions; in the remaining biopsies, the vascular endothelium did not stain. However, 30 min after termination of ILP, a high percentage (76-100%) of the vascular endothelium in normal distant skin and in skin adjacent to melanoma lesions stained with moderate to marked intensity (+ +/+ + +) (Figs. 1, 2A and B, 3A, and 6A).
The endothelial cells near the dermis in the dermis, the normal skin endothelium and aorta lesions in the dermis (E, F). The vascular endothelial cells in the dermis showed a slightly positive staining of IIp, but no staining of IGF-1. Twenty-four hours after topical application of IGF-1, 24% of the microvascular vessels showed E-Selectin staining.

The presence of endothelium-specific expression of E-Selectin in normal skin endothelium (G) and in dermal microvascular vessels (H) was also seen in normal skin and in dermal microvascular vessels. The presence of E-Selectin expression in normal skin endothelium and in dermal microvascular vessels was also seen in normal skin and in dermal microvascular vessels. The presence of E-Selectin expression in normal skin endothelium and in dermal microvascular vessels was also seen in normal skin and in dermal microvascular vessels.
FIG. 3. Immunohistochemical and immunoelectron microscopic analysis of the expression of adhesion molecules in biopsies of isolated limb perfusion (ILP)-treated skin. Asterisks indicate lymph vessels. A: Immunostaining for E-selectin on a frozen section of normal skin, 30 min after perfusion with TNF-α and melphalan (e, epidermis; d, dermis). B: Immunoelectron microscopy. E-selectin is expressed on the luminal and abluminal side (diffuse) of the endothelium of a blood vessel in the dermis of normal skin, 30 min after perfusion with TNF-α and melphalan (E, endothelial cell). C: PECAM-1 immunostaining on a frozen section of normal skin, 30 min after perfusion with TNF-α and melphalan. D: Immunoelectron microscopy. PECAM-1 is expressed on the luminal and abluminal side of the endothelium of a blood vessel in the dermis of normal skin, 30 min after perfusion with TNF-α and melphalan (E, endothelial cell). E: ICAM-1 immunostaining on a frozen section of normal skin, 30 min after perfusion with TNF-α and melphalan. F: Immunoelectron microscopy. ICAM-1 is expressed on the luminal side of a blood vessel in the dermis of normal skin, 30 min after perfusion with TNF-α and melphalan (E, endothelial cell).

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Melanoma and sarcoma cells did not stain. Thirty minutes after termination of ILP, the staining pattern was unchanged both in normal skin and in the melanoma and sarcoma lesions (Fig. 1). By IEM analysis, we did not observe VCAM-1 staining of the cutaneous microvasculature.

**In Vitro Experiments**

Although the use of cultured cells may have limited value in predicting or explaining in vivo mechanisms, we made an attempt to delineate possible synergistic effects of TNF-α and melphalan on the expression patterns of E-selectin, ICAM-1, and VCAM-1 on cultured HUVECs. To mimic the clinical situation most closely, the exposure of HUVECs to TNF-α, melphalan, and perfusate was limited to 1 h. The drug regimen used in vitro was similar to that used in vivo, which is based on the reported synergism of TNF-α with chemotherapy.

Exposure of HUVECs to a pulse of TNF-α, 10 μg/ml for 1 h, resulted in a marked upregulation of E-selectin and ICAM-1 expression (Fig. 4A and D). E-selectin expression reached a maximum after 4 h and decreased within 24 h. ICAM-1 expression reached a maximum after 24 h. The VCAM-1 expression did not differ significantly from control levels (Fig. 4G).

Exposure of HUVECs to a pulse of melphalan, 40 μg for 1 h, did not result in a significant upregulation of E-selectin and VCAM-1 (Fig. 4B and H). Repeated experiments did not reveal a significant pattern of induction of ICAM-1 expression as compared with control levels, but a trend toward an increased expression after 24 h could be observed (Fig. 4E).

Exposure of HUVECs to perfusate (with 4 μg/ml TNF-α and 10 μg/ml melphalan) for 1 h resulted in
E-SELECTIN EXPRESSION

E-selectin is also expressed in vitro (25-26). Neutrophils, CD4+ lymphocytes, monocytes, cosinophils, and basophils also use VCAM-1 and ICAM-1. In vivo interactions however, are more complex (27). Cotran et al. (28), Muro et al. (29-30), and Pober et al. (31-33) described the kinetics of expression of cellular adhesion molecules in natural disease processes in humans and after intra-

**FIG. 5.** Immunohistochemical analysis of the expression of adhesion molecules in biopsies of isolated limb perfusion-treated melanoma metastasis, A-C: Immunostaining for PAL-E on a frozen section of a melanoma metastasis. B-D: Immunostaining for E-selectin on a frozen section of a melanoma metastasis. E: Immunostaining for ICAM-1 on a frozen section of a melanoma metastasis. A and B are high-resolution images of the same sections as shown in A and B, complemented with a staining for a marked upregulation of E-selectin and ICAM-1 expression (Fig. 4C and F). E-selectin levels reached a maximum after 4 h and decreased within 24 h, comparable to those of TNF-α alone. ICAM-1 expression reached a maximum after 16 h. VCAM-1 expression did not differ significantly from control levels (Fig. 4).
In baboons, intradermal injections of TNF-α (or dermal injections of cytokines in baboons (20,33). In baboons, intradermal injections of TNF-α (or TNF-α with IFN-γ) induced a rapid induction of E-selectin expression on the vascular endothelium (which correlated with neutrophil extravasation) and a delayed increase in ICAM-1 (which correlated with the onset of lymphocyte accumulation). In humans, diseased tissues have been examined for the expression of cell-adhesion molecules. E-selectin was present on vascular endothelial cells in the acute inflamed skin (19,34) and on endothelial cells in several tumors characterized by high local concentrations of cytokines (especially TNF-α; 35). Intradermal injections of TNF-α indicate that TNF-α is pro-inflammatory in human skin and modulates inflammatory reaction. Renard et al. (7) investigated biologic response than is observed during an inflammatory process. The authors suggested that the clinically evident preferential sensitivity of the tumor vasculature for the triple-combination therapy could be based on a cascade starting with a selective overexpression of cellular-adhesion molecules by the tumor vascular endothelium. However, a comparison with unexposed and exposed skin from the same patients was not available.

In our study, we sequentially surveyed skin tissue with and without tumor lesions before and after ILP and TNF-α and melphanal, without IFN-γ. We found a marked immunostaining for E-selectin on the vascular endothelium 30 min after termination of perfusion in both normal skin and the melanoma and sarcoma lesions. The percentage of vascular endothelium that became positive for E-selectin was even higher in normal skin than in the tumors (both melanoma and sarcoma). Therefore, a selective overexpression of adhesion molecules on the tumor vasculature after perfusion cannot be the mechanism behind the antitumor effect. It is our opinion that the limited material in the “normal skin” group in the study of Renard et al. (9) may explain the different conclusion reached by these authors.

The rapid increase of E-selectin expression after ILP in both tumor and normal vessels makes this adhesion molecule not a useful marker to discriminate between “normal” and “tumor” vessels, but it may mark those vessels that were actually perfused. In this respect, it is notable that in both melanoma and sarcoma lesions expression of E-selectin was confined to the vascular endothelium in the periphery of the tumor. We therefore speculate that the agents used have not or have poorly reached the tumor center. According to Jain (37,38), aberrant branching and twisting of the vasculature and abnormal high pressure in the interstitial matrix of the tumor center may have contributed to this uneven distribution of the drugs.

We were surprised to find leukostasis and an inflammatory infiltrate in the biopsies of only one patient with melanoma. Expression of an inducible cell-adhesion molecule like E-selectin is apparently not necessarily followed by leukocyte adhesion. This phenomenon may be explained by assuming that the adhesion properties of leukocytes to E-selectin are also dependent on its ligand (39), which may be functionally inactive, as stated by Pober and Cotran (31). Alternatively, inhibitory factors may be present. For example, IL-8 renders neutrophils less able to interact with E-selectin (40), and increased levels of this cytokine (and IL-6) have been demonstrated in the ILP circuit during treatment (41). Soluble adhesion molecules, shed from the cell surface, may counteract leukocyte accumulation as well (42). The presence of these factors was not analyzed in our material. For an optimal monitoring of the duration of E-selectin expression and infiltrate formation in the sarcoma patients, the number of biopsies has to be extended at least to 8–12 h after perfusion. Obviously, this is limited by clinical and ethical restrictions based on the increased infection risk of taking needle biopsies.

The response rate in melanoma-in-transit metastasis after double-drug ILP (CR in six of seven patients) was comparable with results after triple-combination therapy. In contrast to earlier reports, we did not find hemorrhagic and coagulative necrosis in the melanoma lesions within 24 h after perfusion. Sampling bias was minimized by taking multiple biopsies per period per patient. In case of a complete response, biopsies 8 weeks after ILP revealed dermal fibrosis with infiltration by macrophages and lymphocytes. (Biopsies that were taken 8–10 weeks after perfusion are not included in Table 1. These biopsies were formalin-fixed and embedded in paraffin to verify the response to treatment but were not available for immunohistochemistry.)

The presence of regression of melanoma in the absence of granulocytes and hemorrhagic or coag-
The variability in response to ILP and the occurrence of necrosis may imply that the proposed cascade, starting with E-selectin expression, is not the decisive mechanism resulting in regression of the tumor observed in our melanoma patients. The observed regression of the melanoma lesions 8–10 weeks after perfusion may still be immune mediated, a possibility that needs further investigations. The variability in response to ILP and the occurrence of necrosis may be a result of difference in the extent of (neo)vascularization and tumor volume. All the melanoma metastases we analyzed had a diameter ≤0.5 cm (43,44). Obviously, the sensitivity of the tumor for ILP is influenced by more factors than the mere expression of adhesion molecules. For instance, differences in the interstitial environment and in immunoreactivity between tumor and normal tissues may play a role. Further investigations are therefore necessary to explain the exact nature of this selective response.

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