Synergistic Antitumor Response of Interleukin 2 with Melphalan in Isolated Limb Perfusion in Soft Tissue Sarcoma–Bearing Rats

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Abstract

The cytokine interleukin 2 (IL-2) is a mediator of immune cell activation with some antitumor activity, mainly in renal cell cancer and melanoma. We have previously shown that tumor necrosis factor (TNF)-α has strong synergistic antitumor activity in combination with chemotherapeutics in the isolated limb perfusion (ILP) setting based on a TNF-mediated enhanced tumor-selective uptake of the chemotherapeutic drug followed by a selective destruction of the tumor vasculature. IL-2 can cause vascular leakage and edema and for this reason we examined the antitumor activity of a combined treatment with IL-2 and melphalan in our well-established ILP in soft tissue sarcoma–bearing rats (BN175). ILP with either IL-2 or melphalan alone has no antitumor effect, but the combination of IL-2 and melphalan resulted in a strong synergistic tumor response, without any local or systemic toxicity. IL-2 enhanced significantly melphalan uptake in tumor tissue. No signs of significant vascular damage were detected to account for this observation, although the tumor sections of the IL-2– and IL-2 plus melphalan–treated animals revealed scattered extravasation of erythrocytes compared with the untreated animals. Clear differences were seen in the localization of ED-1 cells, with an even distribution in the sham, IL-2 and melphalan treatments, whereas in the IL-2 plus melphalan–treated tumors clustered ED-1 cells were found. Additionally, increased levels of TNF mRNA were found in tumors treated with IL-2 and IL-2 plus melphalan. These observations indicate a potentially important role for macrophages in the IL-2–based perfusion. The results in our study indicate that the novel combination of IL-2 and melphalan in ILP has synergistic antitumor activity and may be an alternative for ILP with TNF and melphalan. (Cancer Res 2005; 65(10): 4300-8)

Introduction

We have shown that isolated limb perfusion (ILP) with tumor necrosis factor (TNF)-α and melphalan is associated with excellent antitumor effects against melanoma (1), large soft tissue sarcomas (2, 3), and various other tumors in the clinical setting (4–6). We have previously shown that the basis for the synergy is, on one hand a significant enhancement of tumor-selective melphalan uptake and on the other hand the subsequent complete destruction of tumor vasculature (2, 7). The enhanced uptake of different cytotoxic agents shown in various limb and liver tumor models in our laboratory prompted us to investigate a number of vasoactive substances for similar potential effects (7–12).

One of these agents is the cytokine interleukin 2 (IL-2) that is known to cause significant changes in vascular permeability and to cause a vascular leakage syndrome when given at high concentrations (13–15). IL-2 is a pleiotropic cytokine that is mainly known as a molecule of central importance in the long-term culture of T lymphocytes and as a mediator of immune cells (15, 16). IL-2, as a single agent, has been shown to have antitumor activity in both animal models (17, 18) and some antitumor activity in mainly renal cell cancer or melanoma patients (19–21).

Here we report on the evaluation of the effects of high concentrations of IL-2 in combination with melphalan in the ILP setting.

Materials and Methods

Chemicals. Human recombinant interleukin 2 (IL-2) was kindly provided by Chiron (Amsterdam, the Netherlands). The content of one vial of lyophilized IL-2 (1 mg per vial, specific activity 18×10^6 IU/mg) was diluted in 1 mL sterile water for injections according to the manufacturer's instructions. Melphalan (Alkeran, 50 mg per vial, Wellcom, Beckenham, United Kingdom) was dissolved in 10 mL of diluent solvent. Further dilutions were made in PBS to a concentration of 2 mg/mL. Fluorescein and FITC conjugated to bovine serum albumin (FITC-BSA) were purchased from Sigma (Zwijndrecht, the Netherlands) and dissolved in PBS to a concentration of 10 mg/mL.

Animals and tumor model. Male inbred Brown Norway rats were used for the soft tissue sarcoma model (BN175). Rats were obtained from Harlan-CPB (Austerlitz, the Netherlands), weighing 250 to 300 g, and were fed a standard laboratory diet ad libitum (Hope Farms, Woerden, the Netherlands). Small fragments (3 mm) of the syngeneic BN175 sarcoma were implanted s.c. in the right hind leg just above the ankle as previously described (8, 10). Tumor growth was recorded by caliper measurement and tumor volume calculated using the formula 0.4 ($A^2 \times B$), where B represents the largest diameter and A the diameter perpendicular to B. Rats were sacrificed if tumor diameter exceeded 25 mm or at the end of the experiment. All animal studies were done in accordance with protocols approved by the committee on Animal Research of the Erasmus MC, Rotterdam, the Netherlands.

Isolated limb perfusion. The perfusion technique was done as described previously (8, 10). Perfusions were done at a tumor diameter of 12 to 15 mm at least 7 days after implantation. During perfusion animals were anaesthetized with Hypnorm and Ketamine (Janssen Pharmaceutica, Tilburg, the Netherlands). The femoral vessels were approached through an incision parallel to the inguinal ligament after systemic heparin administration of 50 IU (Leo Pharmaceutical Products, Weesp, the Netherlands) to prevent coagulation in the collateral circulation and in the perfusion circuit. The femoral artery and vein were cannulated with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer diameter, respectively; Dow Corning, Ann Arbor, MI). Collaterals were temporarily occluded by applying a tourniquet around the groin. An oxygenation reservoir filled with 5 mL Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and a low-flow roller pump (Watson Marlow

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type 505 U, Falmouth, United Kingdom) were included into the circuit. Drugs, 50 μg IL-2 and/or 40 μg melphalan, were added to the Haemaccel reservoir. The roller pump circulated the perfusate at a flow of 2.4 mL/min for 30 minutes. A washout with 5 mL oxygenated Haemaccel was done at the end of the perfusion. During ILP and washout, the hind leg was kept at a constant temperature of 38°C to 39°C by a warm-water mattress applied around the leg. The pH of the perfusate was monitored during ILP with a pH probe in the perfusion reservoir (pH meter HI 8424, Hanna Instruments, Inc., Ann Arbor, MI).

Assessment of tumor response. Tumor size was recorded by daily caliper measurements. The classification of tumor response was progressive disease (PD), increase of tumor volume (>25%); no change (NC), tumor volume equal to volume during perfusion (in a range of −25% and 25%); partial remission (PR), decrease of tumor volume (−25% and −90%); complete remission (CR), tumor volume less than 10% of initial volume (10). To test synergy of IL-2 and melphalan, the tumor response ratio is calculated by dividing the tumor volume at day 0 with the volume at day 8. The ratio of IL-2 alone plus melphalan alone was compared with the ratio of IL-2 plus melphalan (Mann-Whitney *U* test).

***In vitro* response of endothelial and tumor cells to interleukin 2.** Cells isolated from the BN175 soft tissue sarcoma were maintained in cell culture in RPMI 1640 supplemented with 10% FCS and 0.1% penicillin/streptomycin. Medium and supplements were obtained from Life Technologies (Breda, the Netherlands).

Human umbilical vein endothelial cells (HUVEC) were isolated from normal human umbilical cords by the method of Jaffe et. al. (22). Cells were cultured in fibronectin-coated tissue culture flasks in culture medium (human endothelial-SFM, Life Technologies), with 20% newborn calf serum, 10% human serum (Cambrex, Verviers, Belgium), 20 ng/mL basic fibroblast growth factor, and 100 ng/mL epidermal growth factor (Peprotech, London, United Kingdom). Passages 5 to 7 were used for the experiments.

BN175 cells were added in 100-μL aliquots to 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) at a final concentration of 1×10^4 cells per well and allowed to grow as a monolayer. HUVECs were plated in fibronectin-coated 96-well plates at a final concentration of 6×10^3 cells per well. Cells were incubated at 37°C in 5% CO_2 for 72 hours in the presence of various concentrations of IL-2 and melphalan with or without leukocytes in a total volume of 150 μL.

Growth of tumor cells and HUVEC was measured using the sulforhodamine-B (SRB) assay according to the method of Skehan (23). In short, cells were washed twice with PBS, incubated with 10% trichloric acetic acid (1 hour, 4°C), and washed again. Cells were stained with 0.4% SRB (Sigma) for 15 to 30 minutes, washed with 1% acetic acid, and were allowed to dry. Protein-bound SRB was dissolved in TRIS (10 mmol/L, pH 9.4). The absorbance was read at 540 nm. Tumor growth was calculated using the formula: tumor growth = (test well / control) × 100%. The drug concentration reducing the absorbance to 50% of the control (IC_{50}) was determined from the growth curves. The experiments were repeated at least five times.

Preparation of leukocytes. Venous blood from healthy adult volunteers was collected in Na-heparin tubes (Becton Dickinson, Alphen aan den Rijn, the Netherlands). After centrifuging for 20 minutes (1,500 × *g*, room temperature), total WBC fraction was collected, and remaining RBC were lysed with lysis buffer [0.83% NH_4Cl and 10 mmol/L HEPES (pH 7.0)] for 30 minutes at room temperature. After centrifuging for 30 minutes (1,500 rpm, room temperature), the leukocytes were dissolved in HUVEC medium at a concentration of 120×10^4 cells/mL.

Measurement of melphalan in tissue. At the end of the perfusion directly after the washout, the tumor and part of the hind limb muscle were excised. The tissues were immediately frozen in liquid nitrogen to stop metabolism of melphalan and stored at −80°C. Tumor and muscle tissues were homogenized in 2 mL acetonitrile (Pro 200 homogenizer, Pro Scientific, Oxford, CT) and centrifuged at 2,500 × *g*. Melphalan was measured in the supernatant by gas chromatography-mass spectrometry (GC-MS). p-[Bis(2-chloroethyl)amino]-phenylacetic methyl ester was used as an internal standard. Samples were extracted over trifunctional C18 silica columns. After elution with methanol and evaporation, the compounds were derived with trifluoroacetic anhydride and diazomethane in ether. The stable derivates were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single-ion monitoring GC-MS in the positive EI mode described earlier by Tjaden and de Bruijn (24).

Vascular permeability. During ILP, 400 μL FITC-BSA were added to the perfusate. After perfusion, the tumor was excised and frozen in liquid nitrogen. Acetone-fixed frozen sections were fixed for 30 minutes with 4% formaldehyde. After washing with PBS, the slides were incubated for 1 hour with mouse anti–rat-CD31PE (Becton Dickinson) diluted 1:50 in PBS with 5% rat serum. Thereafter, the sections were rinsed with PBS and counterstained with 300 μg/mL Hoechst (Molecular Probes, Leiden, the Netherlands) and mounted with mounting medium containing polyvinyl alcohol (Mowiol 4-88, Fluka, Zwijndrecht, the Netherlands) and 2.5% (w/v) DABCO (Sigma). The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera.

Human umbilical vascular endothelial cell permeability assays. To study the effect of IL-2 on transendothelial monolayer permeability a transwell device (Costar) consisting of an upper chamber with a polycarbonate membrane (6.5 mm, diameter; 0.4 μm, pore size) placed inside a 24-well plate (lower chamber) was used. Confluent HUVECs were trypsinized and 1.2×10^4 cells were seeded on the fibronectin-coated upper chamber. In the lower compartment, 1 mL of HUVEC medium was added. Two days after seeding, nonadhering cells were removed and the medium was replaced with 250 μL of 10 μg/mL IL-2 together with 50 μL FITC-BSA or fluorescein (1 mg/mL). The medium in the lower chamber was replaced with 700 μL of HUVEC medium. At 0.25, 0.5, 1, 2, 4, 8, and 24 hours, 50 μL medium of the lower chamber were taken and fluorescence activity was measured under excitation at 490 nm and emission at 530 nm. A standard curve was prepared with known concentrations of FITC-BSA or fluorescein. Induction of permeability was indicated by a higher concentration of FITC-BSA or fluorescein in the lower chamber of the transwell, relative to untreated controls.

H&E staining. Directly after ILP, tumors were excised, stored in formalin, and embedded in paraffin. The 4-μm sections were stained with H&E using standard procedures. Three or four different tumors in each experimental group were subjected to blind evaluation. At least six slides were examined from each tumor. The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera.

Apoptosis assays: terminal deoxynucleotidyl transferase–mediated nick-end labeling/CD31PE double staining. Apoptotic cell death was detected using the technique of 3′ hydroxy end labeling. A commercially available end-labeling kit (*In situ* Cell Death detection Kit, Fluorescein labeled, Roche, Almere, the Netherlands) was used. Tumor tissues were also stained for endothelial cells to differentiate between apoptosis of the endothelium and apoptosis of tumor cells. Acetone-fixed frozen sections were fixed in 4% paraformaldehyde for 30 minutes and incubated for 1 hour with mouse-anti-rat CD31PE (Becton Dickinson) diluted 1:50 in PBS with 5% rat serum. After washing with PBS the sections were again fixed in 4% paraformaldehyde for 10 minutes and incubated in 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice to allow permeabilization. The slides were incubated with the terminal deoxynucleotidyl transferase–mediated nick-end labeling mixture for 60 minutes at 37°C. After incubation, the slides were rinsed thrice in PBS and counterstained with 300 μg/mL Hoechst (Molecular Probes) for 10 minutes. After washing with PBS, the slides were mounted with mounting medium containing polyvinyl alcohol (Mowiol 4-88, Fluka). The slides were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera.

Immunohistochemistry. After ILP, the tumor was excised and immediately frozen in liquid nitrogen. Immunohistochemical studies were done on acetone-fixed 7-μm cryostat sections. The tumor sections were fixed for 30 minutes with 4% formaldehyde and after rinsing with PBS the endogenous peroxidase activity was blocked by incubation for 5 minutes in methanol/3% H_2O_2. The slides were incubated for 1 hour with 1:50 mouse-anti-rat-CD31, CD4, CD8, antibodies to granulocytes (clone HIS48, Becton Dickinson) or macrophages (ED-1; Serotec, Breda, the Netherlands) diluted in 5% rat serum/PBS. Thereafter, sections were washed with PBS and incubated for 1 hour with goat anti-mouse peroxidase-labeled antibody

(DAKO, ITK Diagnostics BV, Uithoorn, the Netherlands) diluted 1:100 in PBS with 5% rat serum. After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with 3,3′-diaminobenzidine solution (DAB-kit, DAKO) and counterstained lightly with hematoxylin (Sigma).

For quantification of macrophage, $CD4^+$ cell, $CD8^+$ cell and granulocyte infiltration, and microvessel density, two independent persons did blinded analysis. Six representative fields (magnification, 16×) in each slide and three tumors per treatment were evaluated. The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera. For macrophage, T cell and granulocyte infiltration the total number of positive cells per field of interest were counted. For the microvessel quantification, the area of vessels per field of interest was measured in calibrated digital images (Research Assistant 3.0, RVC, Hilversum, the Netherlands) and number of vessels counted.

Reverse transcription-PCR. Total RNA was extracted from frozen tumor tissue using the guanidine isothiocyanate–based TRIzol reagent (Life Technologies) according to the manufacturer's specifications. BN175 cells *in vitro* were treated with medium, 10 μg/mL IL-2, 8 μg/mL melphalan or IL-2 plus melphalan and after 30 minutes of incubation total RNA was extracted. All procedures were carried out with sterile, RNase-free solutions, reagents, and disposables. Total RNA was quantified by spectrophotometric analysis at wavelengths of 260 and 280 nm. To assure the quality of the RNA isolates, samples were analyzed by electrophoresis in agarose gel.

A volume of 20 μL containing 1.0 μg of total RNA of each sample was used for generation of cDNA with Omniscript Reverse Transcriptase (Qiagen, Leusden, the Netherlands) and oligo $d(T)_{16}$ (Life Technologies). After incubation at 42°C for 1 hour, the samples were heated for 5 minutes at 93°C to terminate the reaction. Titanium Taq DNA polymerase (Becton Dickinson) was used for the PCRs and 1.5 μL of cDNA per 37.5 μL of reaction mixture was used. The primers were purchased from Life Technologies and primer sequences are shown in Table 1. β-Actin was used as an internal standard. PCRs were done on a Biometra T-gradient PCR machine using the following variables: initial denaturation at 94°C for 5 minutes followed by a maximum of 40 cycles of 94°C for 45 seconds, annealing for 45 seconds (for temperatures, see Table 1), and extension 72°C for 1 minute and a final extension step at 72°C for 7 minutes. The resulting DNA fragments were electrophoretically separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A 100-bp ladder was used as the standard.

Semiquantitative reverse transcription-PCR. Total RNA isolation, cDNA preparation and reverse transcription-PCR (RT-PCR) were done as described above (see RT-PCR). Semiquantification of cytokine expression was carried out as followed, every two cycles, 5 μL of PCR product were collected and the samples were electrophoretically separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. The threshold cycle was determined as the cycle where the visible band of a specific PCR product first appeared on the gel. Intensities of the PCR product bands were determined by ImageJ v1.34 software (W. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD) and normalized for β-actin.

Statistical analysis. Results were evaluated for statistical significance with the Mann-Whitney *U* test. *P*s < 0.05 were considered statistically significant. Calculations were done on a personal computer using GraphPad Prism v3.0 and SPSS v11.0 for Windows 2000.

Results

Tumor response in interleukin 2–based isolated limb perfusion. To evaluate the antitumor activity of melphalan when combined with IL-2 in an ILP, soft tissue sarcoma–bearing rats were perfused with the agents alone or combined. Sham perfusion with Haemaccel alone resulted in progressive disease in all animals (Fig. 1; Table 2), whereas application of melphalan resulted in a slight inhibition of the tumor growth, with a tumor response rate of 17% (PR and CR combined). Progressive disease was also seen in all animals perfused with 50 μg IL-2. Perfusion with IL-2 plus melphalan resulted in a strong synergistic antitumor response and tumor response reaching 67% ($P < 0.05$ compared with melphalan alone). We statistically proved the IL-2 plus melphalan synergy ($P < 0.02$). No obvious regional or systemic toxicity was observed in any of the treatments.

Table 1. RT-PCR primers for the immune-related genes and for *β-actin*, which was used as a housekeeping gene

Gene	Primers	Annealing temperature (°C)	Product size (bp)
β-actin	f, 5′-ATGGATGACGATATCGCTG-3′ r, 5′-ATGAGGTAGTCTGTCAGGT-3′	60	569
IL-6	f, 5′-GACTTCACAGAGGATACC-3′ r, 5′-TAAGTTGTTCTTCACAAACTCC-3′	55	294
GRO/CINC-A	f, 5′-GAAGATAGATTGCACCGATG-3′ r, 5′-CATAGCCTCTCACACATTTC-3′	57	367
IL-10	f, 5′-TGACAATAACTGCACCCACTT-3′ r, 5′-TCATTCATGGCCTTGTAGACA-3′	60	402
IL-12	f, 5′-TCATCAGGGACATCATCAAACC-3′ r, 5′-CGAGGAACGCACCTTTCTG-3′	65	210
TNF-α	f, 5′-TACTGAACTTCGGGGTGATCGGTCC-3′ r, 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	60	295
IFN-γ	f, 5′-GCCTCCTCTTGGATATCTGG-3′ r, 5′-GTGCTGGATCTGTGGGTTG-3′	60	239
MCP-1	f, 5′-ATGCAGGTCTCTCTGTCACG-3′ r, 5′-CTAGTTCTCTGTCATACT-3′	57	446
MIP-2	f, 5′-GGCACAATCGGTACGATCCAG-3′ r, 5′-ACCCTGCCAAGGGTTGACTTC-3′	55	287
TGF-β1	f, 5′-TGGAAGTGGATCCACGAGCCCAAG-3′ r, 5′-GCAGGAGCGCACGATCATGTTGGAC-3′	55	240

Abbreviations: f, forward primer; r, reverse primer.

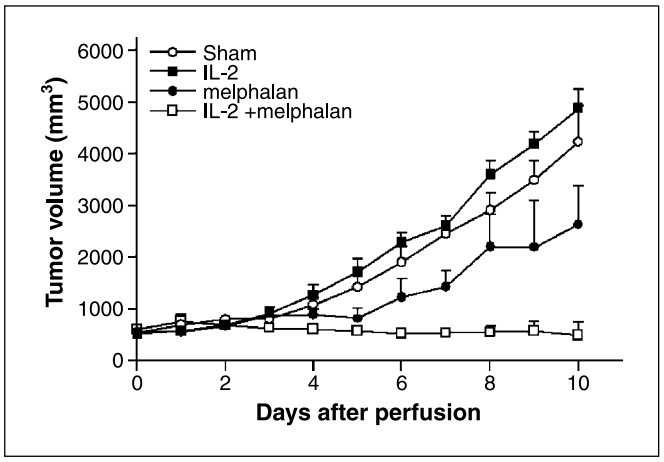

Figure 1. Tumor volumes of the s.c. implanted soft tissue sarcoma BN175 after ILP with perfusate alone, 50 μg IL-2, 40 μg melphalan, or a combination of IL-2 and melphalan. *Points,* means of tumor volumes; *bars,* ±SE. Number of rats per group is shown in Table 2.

Direct effect of interleukin 2 and melphalan on BN175 and endothelial cells. *In vitro* experiments were done to define whether direct cytotoxicity contributed to the improved tumor response. Because the target can be tumor vascular endothelial cells as well as tumor cells, both HUVECs and BN175 cells were tested. No direct cytotoxicity could be observed when BN175 cells or HUVECs were exposed to concentrations of IL-2 up to 10 μg/mL. Exposure of BN175 cells or HUVECs to melphalan resulted in a response curve with an IC_{50} of 0.25 and 11.4 μg/mL, respectively. Addition of IL-2 to melphalan did not alter the IC_{50} of melphalan in both cell types. Incubation of HUVEC with IL-2 did not change the typical cobblestone-shape of these cells. IL-2 had also no additive effect on HUVECs when coincubated with leukocytes (data not shown).

Tissue concentrations of melphalan. Accumulation of melphalan in tumor and normal muscle tissue was determined. A highly significant tumor-selective increase of melphalan uptake was observed in the rats treated by an ILP with IL-2 and melphalan in comparison with rats treated with an ILP with melphalan alone. Figure 2 shows a 3.7-fold increase in melphalan concentration in tumor tissue after perfusion with IL-2 plus melphalan ($n = 4$) in comparison with perfusion with melphalan alone ($n = 4$; $P < 0.01$). Importantly, IL-2 had no effect on the uptake of melphalan by muscle tissue.

Table 2. Tumor response in soft tissue sarcoma–bearing rats after ILP with IL-2 and melphalan over a total period of 10 days

Treatment	Response rate* (%)				
	PD	NC	PR	CR	RR
Sham ($n = 8$)	100	—	—	—	0
IL-2 ($n = 9$)	100	—	—	—	0
Melphalan ($n = 6$)	66	17	17	—	17
IL-2 + melphalan ($n = 8$)	22	11	56	11	67†

Abbreviation: RR, response rate (PR plus CR).
*Responses were scored as described in Materials and Methods.
†$P < 0.05$ compared with melphalan alone.

Vascular permeability and damage by interleukin 2. Because we observed an increased accumulation of melphalan especially in tumor tissue after ILP with IL-2 and melphalan, we investigated the effect of IL-2 on the tumor vascular lining in more detail. First, we looked at extravasation of a larger tracer molecule (FITC-BSA) into the tumor tissue. During the perfusion, FITC-BSA was added to the perfusate and after ILP tumors were excised and frozen sections were stained with CD31PE to visualize blood vessels. We saw no increased vascular permeability for this molecule in the IL-2 plus melphalan group compared with the sham group (data not shown). Most of the FITC-BSA was still present in the blood vessels and hardly any extravasation of the albumin had taken place. For this we hypothesized that permeability of the relatively small melphalan (0.3 kDa) molecule is differently affected as compared with FITC-BSA (66.4 kDa).

These results were confirmed *in vitro* in which we assessed the capacity of IL-2 to induce permeability in endothelial cell monolayers. A transwell insert with only fibronectin coating and no cells was used to determine the maximum passage of FITC-BSA or fluorescein across the membrane. Incubation of HUVECs with 10 μg/mL IL-2 did not cause an increase in monolayer permeability for FITC-BSA in a period of 24 hours (data not shown). These experiments were repeated with fluorescein, a smaller molecule. Incubation with IL-2 resulted in a 1.6-fold increased permeability compared with untreated cells after 15 minutes of exposure and incubation times of 1 hour or longer showed no increased permeability anymore (Fig. 3). In conclusion, there was a transient effect of IL-2 on the permeability of endothelial cells *in vitro* for small molecules like fluorescein and not for proteins like BSA. This could explain why an increased melphalan uptake *in vivo* directly after ILP was seen and no increased permeability of FITC-BSA *in vivo*.

pH measurement in perfusate. Before and during perfusion, the pH of the perfusate was measured. The pH of Haemaccel is 6.9 and oxygenation lowered the pH to 6.2. Directly after start of the perfusion the pH increased up to 6.5 ± 0.3. Different treatments did not have an effect on the pH of the perfusate and the pH at the end of the perfusion was 6.9 ± 0.1 for all four treatments.

H&E staining and apoptosis *in vivo*. Histopathologic examination was done on the tumors from animals treated with sham, IL-2, melphalan or IL-2 plus melphalan to evaluate damage to the endothelial lining of tumor vessels. The animals were autopsied

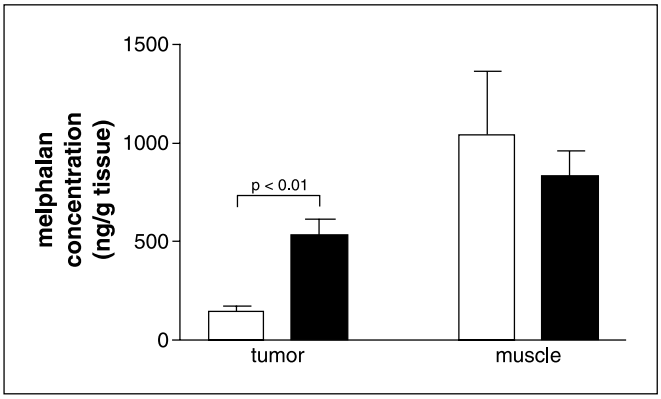

Figure 2. Accumulation of melphalan in soft tissue sarcoma BN175 and muscle during ILP. Rats were perfused with 40 μg melphalan (*open column*) or 50 μg IL-2 plus 40 μg melphalan (*closed column*), after which tumor and muscle were excised and total melphalan content determined as described in Materials and Methods. *Columns,* means of six rats; *bars,* ±SD.

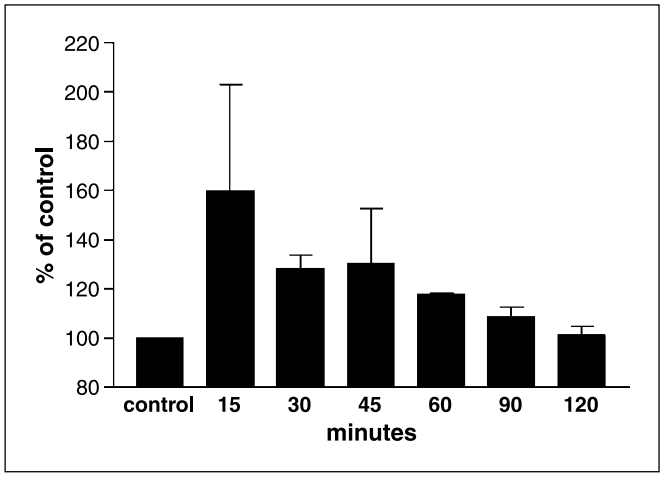

Figure 3. Effect of IL-2 on HUVEC monolayer permeability. HUVECs were cultured on the filter of a transwell unit for 48 hours before the addition of fluorescein-containing medium (*control*) or IL-2 (see Materials and Methods). The amount of fluorescein in the lower compartment was measured for a period of 2 hours. Values are from two experiments, each done in duplicate. *Columns,* means; *bars,* ±SD.

directly after ILP and the tumor slides were stained with hematoxylin. The tumor sections of the IL-2– and IL-2 plus melphalan–treated animals revealed scattered extravasation of erythrocytes compared with the sham- and melphalan-treated animals, although the endothelial lining seemed mostly to be intact (Fig. 4). In the IL-2 and the IL-2 plus melphalan treatment, there was a small increase in edema in the tumor in comparison with the other two groups. Because the BN175 tumor is a fast growing tumor, necrotic areas were seen in all four treatments (data not shown). At this immediate post-ILP time point, there seemed no difference in the number and size of the necrotic areas between the treatments.

The subtle changes in vascular leakage and damage to the tumor vessels evoked by IL-2 were further confirmed by double staining of tumor sections for apoptosis and for CD31 expression. Only a few apoptotic tumor and endothelial cells were detected in both the sham- and the IL-2–treated rats, without differences between the two groups (data not shown). These results indicate that IL-2 has a much less pronounced effect on the tumor vasculature when compared with TNF, which inflicts massive hemorrhagic necrosis when used in ILP (25).

Assessment of tumor vascular functionality. The increased uptake of melphalan might correlate with the functionality of the

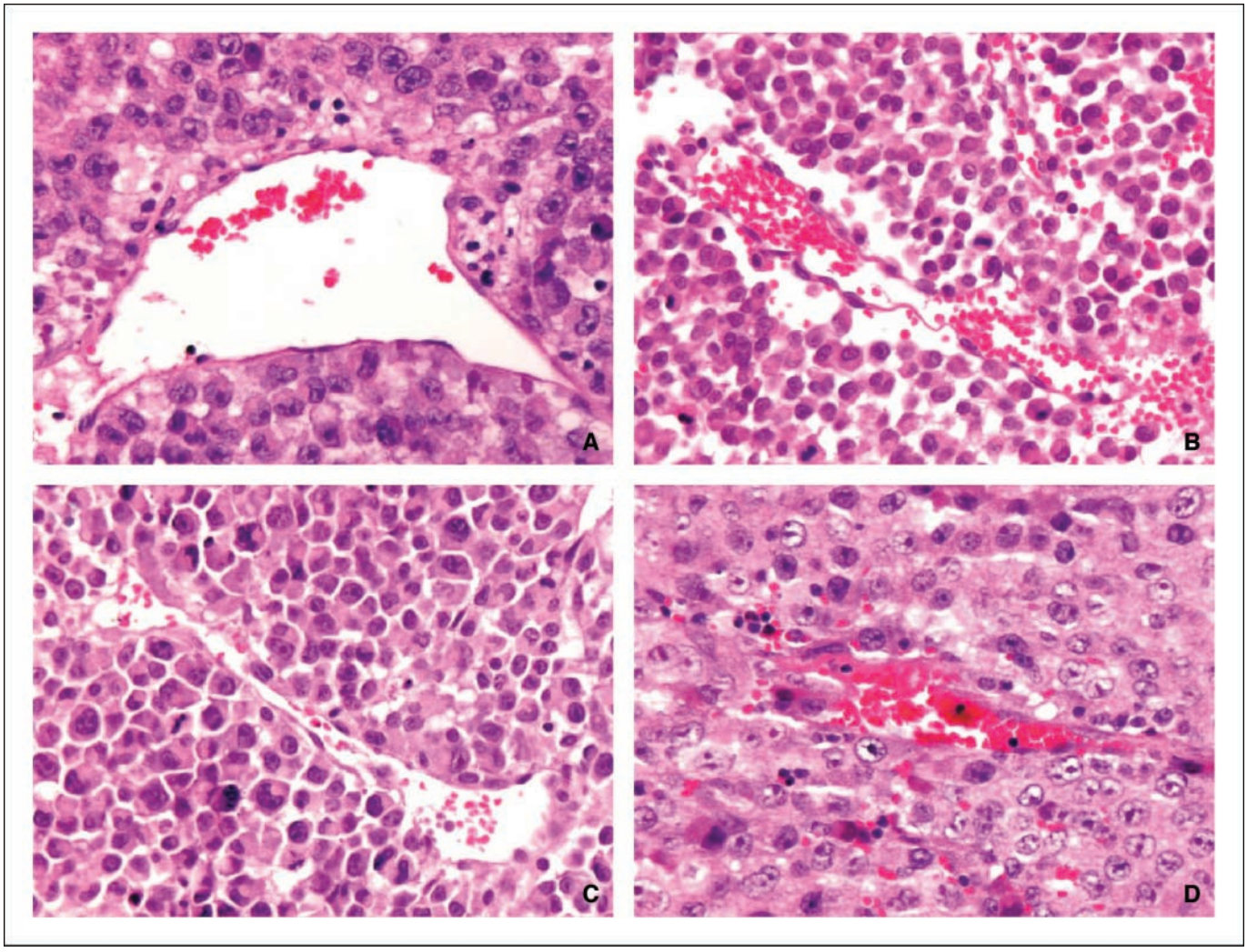

Figure 4. Paraffin sections of BN175 tumor tissue after ILP, H&E stained. Sham (*A*), 50 μg IL-2 (*B*), 40 μg melphalan (*C*), and IL-2 plus melphalan (*D*). Original magnification, ×40.

tumor-associated vasculature. Quantification of the microvessel density and functionality was done by immunohistochemical staining of endothelial cells. The number of vessels as well as vessel area was measured. The area per vessel is computed by dividing the total area of vessels by the number of vessels. There was no significant difference between the treatments in the number of vessels, area endothelium, or the area per vessel (Table 3).

Tumor infiltration of leukocytes and macrophages. *In vitro*, IL-2 seems to have a small and transient effect on endothelial cells and no effect on tumor cells, which is also seen with TNF. We hypothesized that IL-2 stimulates immune cells to contribute to the increased vascular leakage. To see if there is an increased infiltration of leukocytes into the tumor tissue, tumors were excised directly after isolated perfusion with sham, IL-2, melphalan or IL-2 plus melphalan. Immunohistochemical staining for CD4 and CD8 was done on frozen sections. There were hardly any CD4-positive cells present in all four treatments (Table 3). The number of infiltrating CD8 cells was much higher compared with the number of CD4 cells. However, no clear differences in the amount of infiltrating CD8 cells were seen between the four groups.

Infiltrated granulocytes were detected in tumor tissue in slightly larger numbers than CD8 cells. ILP with melphalan resulted in a decreased number of granulocytes compared with sham perfusions ($P = 0.05$). Addition of IL-2 to the sham perfusion increased the number of infiltrating cells and addition of IL-2 to melphalan ILP also resulted in an increase in infiltrating granulocytes ($P = 0.05$). However, no increased infiltration of granulocytes in the IL-2 plus melphalan group compared with sham perfusions was found. In none of the treatments, a difference in distribution pattern of granulocytes was found.

Macrophages were present is all treated and untreated tumors in larger proportions than T cells and granulocytes, without differences between the treatments. However, clear differences were seen in the localization of ED-1 cells, with an even distribution in the treatments sham, IL-2 and melphalan, whereas in the IL-2 plus melphalan group clustered ED-1 cells were found (Fig. 5).

Cytokine expression in tumor tissue and tumor cells *in vitro*. The different macrophage distribution after perfusion with IL-2 and melphalan indicates possible macrophage activation. These activated macrophages could produce nonspecific effector molecules like cytokines and reactive oxygen and nitrogen intermediates, all of which exhibit potent antitumor properties. Tumor biopsies were obtained directly after ILP and RNA extracted from these samples was amplified to create an overview of cytokine profile in the tumor microenvironment and the effect of treatment on this profile.

All cytokines tested were expressed in sham-treated tumors (data not shown). In these tumors, transforming growth factor-β1 (TGF-β1) shown to have the highest expression level followed by MCP-1 and expression of TNF was the lowest compared with the other cytokines tested. Strikingly, only for TNF expression a response to IL-2 treatment was observed. The threshold cycle of TNF expression in IL-2– and IL-2 plus melphalan–treated tumors was five cycles lower than that of sham-treated tumors ($P < 0.05$). Integrated density was measured and TNF mRNA levels were expressed as a ratio of TNF to β-actin (Fig. 6). A 5.7-fold increase in TNF mRNA expression was found in tumor tissue treated with IL-2 plus melphalan compared with sham treatment ($P < 0.05$). IL-2 ILP caused a 3.2-fold increased TNF mRNA expression compared with sham ($P < 0.05$), whereas melphalan ILP had no effect on TNF mRNA expression.

To investigate which cytokines the tumor cells produced, RNA was isolated from BN175 tumor cells *in vitro*. Cells were also incubated with 10 μg/mL IL-2 and 8 μg/mL melphalan or the combination for 30 minutes. Clear differences were seen between cytokine expression levels *in vivo* and *in vitro*. mRNA expression in tumors showed higher levels of IL-12, MCP-1 and TGF-1 than tumor cells *in vitro*. Levels of the other cytokines tested were comparable. Treatment with IL-2 and/or melphalan had no effect on the cytokine expression of tumor cells *in vitro* (data not shown).

Discussion

In this study, we have examined the antitumor activity of a combined treatment with IL-2 and melphalan in an isolated limb

Table 3. Microvessel density, area of the vessels, and tumor infiltration after isolated perfusion with sham, IL-2, melphalan, or IL-2 plus melphalan

	Sham	IL-2	Melphalan	IL-2 + melphalan
No. vessels*	33 ± 9	64 ± 24	22 ± 2	29 ± 5
Area of endothelium†	6.8 ± 0.6	9.2 ± 1.3	5.9 ± 0.9	6.4 ± 0.8
Area endothelium/vessel‡	0.30 ± 0.09	0.16 ± 0.04	0.28 ± 0.03	0.24 ± 0.02
CD4§	0.4 ± 0.2	2.4 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
CD8§	51 ± 7	36 ± 10	67 ± 47	37 ± 10
Granulocytes§	60 ± 4	79 ± 4‖	48 ± 3‖	63 ± 4¶
Macrophages§	210 ± 7	222 ± 52	201 ± 20	189 ± 20

NOTE: Directly after ILP, the tumors were excised and frozen sections were stained for granulocytes, macrophages, and CD31-, CD4-, and CD8-positive cells. At least two animals per group and six fields of interest per tumor were evaluated. Average ± SE is shown.

*No. vessels per field of interest.

†% Total vessel area per field of interest.

‡Area per vessel.

§Amount of positive cells per field of interest.

‖$P = 0.05$ compared with sham treatment.

¶$P = 0.05$ compared with melphalan treatment.

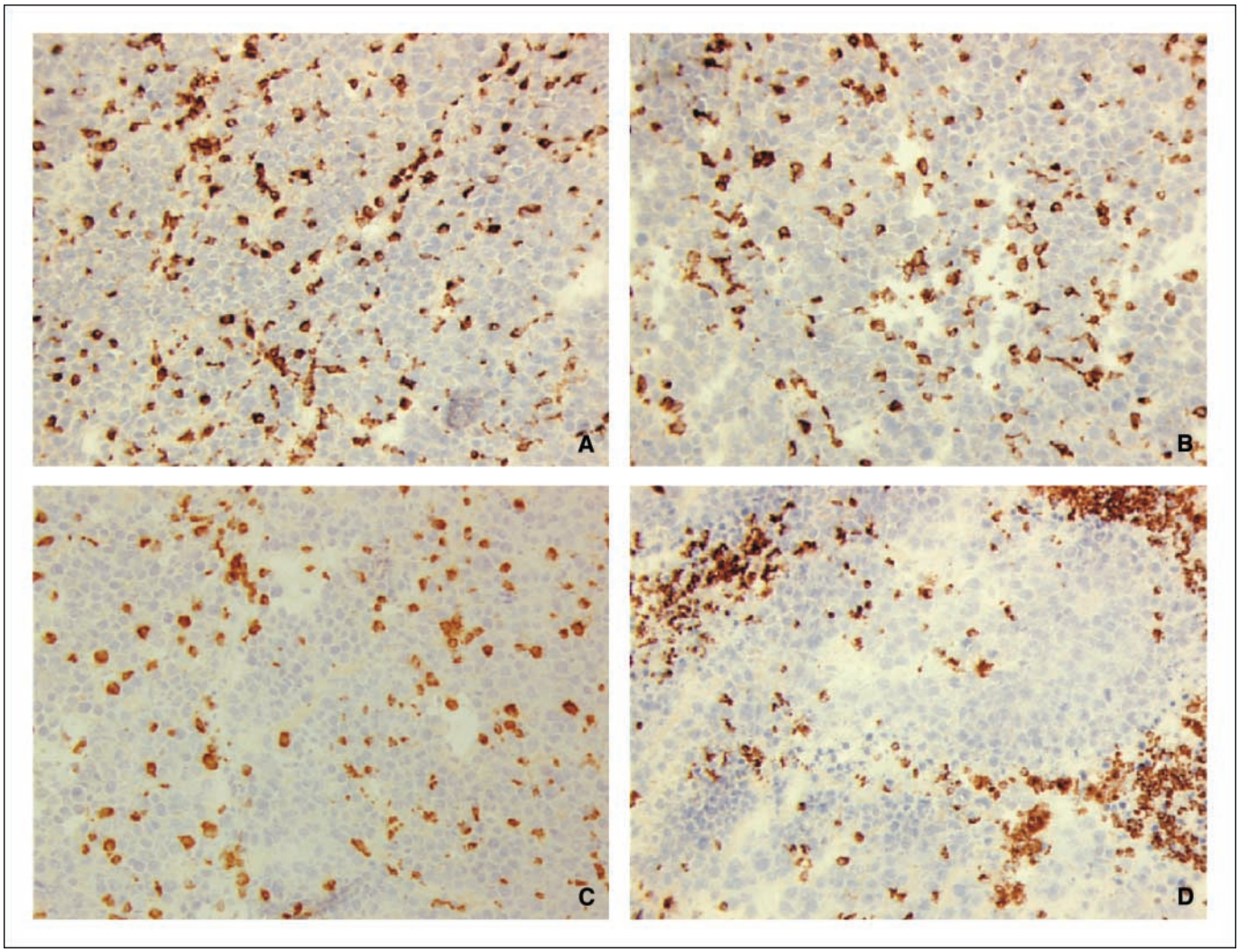

Figure 5. Representative pictures of macrophage localization in BN175 tumor. Rats were perfused with sham (*A*), 50 μg IL-2 (*B*), 40 μg melphalan (*C*), and IL-2 plus melphalan (*D*), after which tumors were excised and an immunohistochemical staining for ED-1–positive cells was done on frozen tumor sections. Treatment with IL-2 plus melphalan resulted in a redistribution of macrophages and clustered ED-1 cells were found. Original magnification, 16×.

perfusion in soft tissue sarcoma–bearing rats. We show for the first time that ILP with the combination of IL-2 and melphalan in the BN175 tumor resulted in a strong synergistic tumor response. The tumor response (PR and CR combined) of 67% was much higher than the tumor response of melphalan alone (17%), whereas progressive disease was seen in all animals treated with IL-2 alone. These results are comparable with those of our previous study with TNF and melphalan, where we found similar synergy for the combination of TNF and melphalan (8, 10). Importantly, the application of high-dose IL-2 in ILP was without any local or systemic toxicity indicating possible translation of this cytokine to loco-regional settings in the clinic.

In different animal models, IL-2 as a single agent has been shown to have antitumor activity (17, 18). In the systemic setting, this requires high doses and multiple dosing, which is associated with serious systemic toxicity with hypotension, massive vascular leakage syndrome, and multiple organ failure leading to death (13–15, 26). The advantage of a loco-regional application (i.e., an ILP) is the minimal systemic exposure while maintaining a high therapeutic dose locally. For this reason, we hypothesized that IL-2 could be a good candidate to be used in a melphalan-based ILP. IL-2 is known not to have direct antitumor effects and thus seems an unlikely candidate to be used in the isolated limb perfusion setting. However, we speculated that IL-2 with its multiple effects could well affect the pathophysiology of large tumors in a similar way as TNF and might thus significantly potentiate the distribution and uptake of melphalan throughout the tumor. A critical step for TNF-based ILP is the accurate and real-time monitoring of systemic leakage with the aim of avoiding severe systemic TNF-mediated toxicity. Because IL-2 is used in a systemic clinical setting, we hypothesized that IL-2 is a potentially safer drug than TNF and therefore a useful alternative for ILP with TNF. Moreover, the lack of toxicity warrants exploration of IL-2 in loco-regional treatment of liver cancer, in which TNF cannot be used effectively due to its inherent dose-limiting liver toxicity.

Here we show that IL-2 alone in the ILP-setting had no effect on tumor growth, in spite of the high dosage we were using. A strong synergistic tumor response was observed when IL-2 was combined with melphalan. A set of experiments to investigate potential mechanisms behind the observed synergy between IL-2

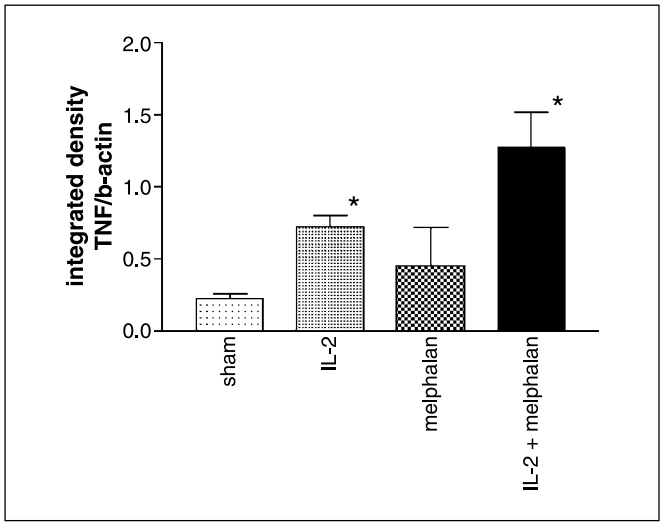

Figure 6. Semiquantitative RT-PCR. BN175-bearing rats were treated with buffer alone, 50 μg IL-2, 40 μg melphalan, or a combination of IL-2 and melphalan, and directly after ILP, the tumors were excised. RNA was isolated and RT-PCR with TNF primers was carried out. Integrated density of the PCR bands was measured and TNF mRNA levels are presented as a ratio of TNF to β-actin. *Columns,* means of three animals per group; *bars,* ±SE. *, $P < 0.05$ compared with sham (Mann-Whitney *U* test).

and melphalan was done both *in vitro* and *in vivo*. We showed that IL-2 did not have an effect on the proliferation or morphology of HUVECs or BN175 tumor cells *in vitro*. We speculate that the improved antitumor effect shown *in vivo* is probably not caused by a direct cytotoxic effect on tumor or endothelial cells.

IL-2 is a 15-kDa glycoprotein produced by antigen-activated T lymphocytes that plays a varied and critical role in immunoregulation. IL-2 binds to the IL-2 receptor and the IL-2R is expressed not only on hematopoietic cells but also on nonhematopoietic cells. Different reports have indicated the presence of IL-2 receptors on cells in head and neck squamous cell carcinoma (27) and different human melanomas (28). We evaluated the direct effect of IL-2 on endothelial cells. We did not see an effect on the proliferation of HUVECs, nor morphologic changes. Holzinger et al. showed that HUVECs possess low numbers of IL-2 receptor, although IL-2 had no effect on the proliferation of the endothelial cells neither on the typical cobblestone-shape morphology of the cells (29). This in contrast with the study of Hicks et al. where they showed that HUVECs do proliferate in response to IL-2 (30). To test whether IL-2 had an indirect effect on HUVECs, we cocultured leukocytes with HUVECs and treated them with different concentrations of IL-2. We showed that there was no effect on proliferation of endothelial cells nor did we observe morphologic changes.

Systemic IL-2 administration is often complicated by significant capillary leakage, with consequent extravasation of interstitial fluid and plasma proteins (14, 15, 26). In this study, we evaluated whether IL-2 could cause capillary leakage in the tumor and therefore enhance the delivery of melphalan at the tumor site. We showed that IL-2 caused a 3.7-fold augmented accumulation of melphalan specifically in tumor tissue, which correlated closely with the enhanced tumor responses. This increase could very well explain the improved efficacy, as ILP with IL-2 alone did not induce any tumor response. The 3.7-fold increase in local melphalan results in a shift from a hardly effective dose of 0.14 μg/mL to an effective dose of 0.53 μg/mL, when translated to the *in vitro* cytotoxicity profile of melphalan on BN175 tumor cells. Taken into account that we expect a heterogeneous distribution of melphalan especially around the tumor vessels in the well-perfused region of the tumor, actual local drug levels are likely to be even higher. Furthermore, IL-2 did not have an effect on the accumulation of melphalan in muscle tissue, indicating that IL-2 works specifically on the tumor-associated vessels.

The mildly acidic condition of the perfusate (pH 6.2) might enhance the antitumor effect of melphalan as suggested by a study of Kelley et al. (31). Others showed that hypoxia and acidosis both *in vitro* and *in vivo* are able to augment the cytotoxicity of melphalan (32, 33). Addition of IL-2 did not have an effect on the pH of the perfusate. The final pH for all treatments was 6.9.

We showed in a previous study that TNF comparably augmented the accumulation of chemotherapeutic drugs specifically in tumor tissue 4- to 6-fold (7, 11). As the augmented melphalan accumulation in tumor tissue induced by IL-2 correspond with our observations in TNF-based ILP, we expected comparable histologic changes. However, the H&E slides of tumor tissue after IL-2–based ILP showed a much less pronounced extravasation of erythrocytes when compared with TNF-based ILP (25). However, in IL-2– and IL-2 plus melphalan–treated tumors, although the endothelial lining seemed intact, scattered extravasation of erythrocytes was observed next to locally increased edema. These findings indicate that IL-2 has a much more subtle effect on the endothelial lining compared with TNF. Moreover, IL-2 did not increase the permeability to FITC-BSA *in vivo*. In addition, *in vitro* endothelial permeability for FITC-BSA was not increased when HUVECs were treated with IL-2, whereas an enhanced permeability of 1.6-fold for fluorescein was seen after 15 minutes of incubation with IL-2. An explanation can be that FITC-BSA is a much larger molecule than fluorescein (molecular weight 66.400 and 332 Da, respectively) and the molecular weight of melphalan (305 Da) is comparable with fluorescein. The lack of a strong tumor vascular effect was confirmed by staining of tumor section for apoptosis. We could only detect few apoptotic tumor and endothelial cells and no differences between sham- and IL-2–based ILP were noted. The increased tumor accumulation of melphalan might be correlated with the vessel functionality of the tumors. However, we did not see any differences between the treatments in the vessel area.

As IL-2 did not seem to have an effect on endothelial or tumor cells directly, we hypothesized that immune cells are involved. We did immunohistochemical stainings on tumor tissue collected directly after ILP. We did not see an effect on the number of infiltrating CD4 or CD8 cells. A reason why we do not see more pronounced differences in the amount of infiltrating cells between the treatments could be explained by the time point at which tissues were collected, after only 30 minutes of treatment. None of the perfusions had an effect on the number of infiltrating macrophages, but clear differences were seen in the localization of macrophages. After ILP with IL-2 and melphalan, clustered macrophages were present, whereas in the other treatments, macrophages were evenly distributed. Lipopolysaccharide, IFNγ, and IL-2 are established as activating agents for monocytes/macrophages. Activated monocytes/macrophages produce cytokines (such as TNF) and free radicals (superoxide and nitric oxide) that have cytotoxic effects on tumor cells (34–37). We showed in tumor

tissue treated with IL-2 alone or IL-2 plus melphalan a 5.7-fold increased expression of TNF, whereas *in vitro* stimulation of tumor cells did not result in an increased TNF expression. These results indicate that activated macrophages could play a role in the antitumor response of IL-2–based ILP. Additional studies are ongoing to further elucidate the mechanism.

In conclusion, IL-2 in a melphalan-based ILP is causing a strong synergistic antitumor response in soft tissue sarcoma BN175. Importantly, the addition of IL-2 inflicted no toxicity locally or systemically. The results in our study indicate that the novel combination of IL-2 and melphalan in an ILP can be of value and therefore possibly a useful alternative for ILP with TNF and melphalan, or as a novel candidate for isolated hepatic perfusion.

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References

1. Lienard D, Lejeune FJ, Ewalenko P. In transit metastases of malignant melanoma treated by high dose rTNF α in combination with interferon-γ and melphalan in isolation perfusion. World J Surg 1992;16:234–40.

2. Eggermont AM, Schraffordt KH, Lienard D, et al. Isolated limb perfusion with high-dose tumor necrosis factor-α in combination with interferon-γ and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. J Clin Oncol 1996;14:2653–65.

3. Eggermont AM, Schraffordt KH, Klausner JM, et al. Isolated limb perfusion with tumor necrosis factor and melphalan for limb salvage in 186 patients with locally advanced soft tissue extremity sarcomas. The cumulative multicenter European experience. Ann Surg 1996; 224:756–64.

4. Bickels J, Manusama ER, Gutman M, et al. Isolated limb perfusion with tumour necrosis factor-α and melphalan for unresectable bone sarcomas of the lower extremity. Eur J Surg Oncol 1999;25:509–14.

5. Eggermont AM, de Wilt JH, ten Hagen TL. Current uses of isolated limb perfusion in the clinic and a model system for new strategies. Lancet Oncol 2003;4:429–37.

6. Olieman AF, Lienard D, Eggermont AM, et al. Hyperthermic isolated limb perfusion with tumor necrosis factor α, interferon γ, and melphalan for locally advanced nonmelanoma skin tumors of the extremities: a multicenter study. Arch Surg 1999;134: 303–7.

7. de Wilt JH, ten Hagen TL, de Boeck G, van Tiel ST, de Bruijn EA, Eggermont AM. Tumour necrosis factor α increases melphalan concentration in tumour tissue after isolated limb perfusion. Br J Cancer 2000;82: 1000–3.

8. de Wilt JH, Manusama ER, van Tiel ST, van Ijken MG, ten Hagen TL, Eggermont AM. Prerequisites for effective isolated limb perfusion using tumour necrosis factor α and melphalan in rats. Br J Cancer 1999;80:161–6.

9. Manusama ER, Stavast J, Durante NM, Marquet RL, Eggermont AM. Isolated limb perfusion with TNF α and melphalan in a rat osteosarcoma model: a new antitumour approach. Eur J Surg Oncol 1996;22:152–7.

10. Manusama ER, Nooijen PT, Stavast J, Durante NM, Marquet RL, Eggermont AM. Synergistic antitumour effect of recombinant human tumour necrosis factor α with melphalan in isolated limb perfusion in the rat. Br J Surg 1996;83:551–5.

11. van Der Veen AH, de Wilt JH, Eggermont AM, van Tiel ST, Seynhaeve AL, ten Hagen TL. TNF-α augments intratumoural concentrations of doxorubicin in TNF-α-based isolated limb perfusion in rat sarcoma models and enhances anti-tumour effects. Br J Cancer 2000;82:973–80.

12. van Etten B, de Vries MR, van Ijken MG, et al. Degree of tumour vascularity correlates with drug accumulation and tumour response upon TNF-α-based isolated hepatic perfusion. Br J Cancer 2003;88:314–9.

13. Epstein AL, Mizokami MM, Li J, Hu P, Khawli LA. Identification of a protein fragment of interleukin 2 responsible for vasopermeability. J Natl Cancer Inst 2003;95:741–9.

14. Siegel JP, Puri RK. Interleukin-2 toxicity. J Clin Oncol 1991;9:694–704.

15. Winkelhake JL, Gauny SS. Human recombinant interleukin-2 as an experimental therapeutic. Pharmacol Rev 1990;42:1–28.

16. Morgan DA, Ruscetti FW, Gallo R. Selective *in vitro* growth of T lymphocytes from normal human bone marrows. Science 1976;193:1007–8.

17. Baselmans AH, Koten JW, Battermann JJ, Van Dijk JE, Den Otter W. The mechanism of regression of solid SL2 lymphosarcoma after local IL-2 therapy. Cancer Immunol Immunother 2002;51:492–8.

18. Den Otter W, De Groot JW, Bernsen MR, et al. Optimal regimes for local IL-2 tumour therapy. Int J Cancer 1996;66:400–3.

19. Eton O, Rosenblum MG, Legha SS, et al. Phase I trial of subcutaneous recombinant human interleukin-2 in patients with metastatic melanoma. Cancer 2002;95: 127–34.

20. Negrier S, Escudier B, Lasset C, et al. Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma. Groupe Francais d'Immunotherapie. N Engl J Med 1998;338:1272–8.

21. Yang JC, Sherry RM, Steinberg SM, et al. Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer. J Clin Oncol 2003;21:3127–32.

22. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745–56.

23. Skehan P, Storeng R, Scudiero D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–12.

24. Tjaden UR, de Bruijn EA. Chromatographic analysis of anticancer drugs. J Chromatogr 1990;531:235–94.

25. Nooijen PT, Manusama ER, Eggermont AM, et al. Synergistic effects of TNF-α and melphalan in an isolated limb perfusion model of rat sarcoma: a histopathological, immunohistochemical and electron microscopical study. Br J Cancer 1996;74:1908–15.

26. Atkins MB. Interleukin-2: clinical applications. Semin Oncol 2002;29:12–7.

27. Weidmann E, Sacchi M, Plaisance S, et al. Receptors for interleukin 2 on human squamous cell carcinoma cell lines and tumor *in situ*. Cancer Res 1992;52:5963–70.

28. Rimoldi D, Salvi S, Hartmann F, et al. Expression of IL-2 receptors in human melanoma cells. Anticancer Res 1993;13:555–64.

29. Holzinger C, Weissinger E, Zuckermann A, et al. Effects of interleukin-1, -2, -4, -6, interferon-γ and granulocyte/macrophage colony stimulating factor on human vascular endothelial cells. Immunol Lett 1993;35:109–17.

30. Hicks C, Cooley MA, Penny R. Investigation of interleukin 2 receptors on human endothelial cells. Growth Factors 1991;5:201–8.

31. Kelley ST, Menon C, Buerk DG, Bauer TW, Fraker DL. Acidosis plus melphalan induces nitric oxide-mediated tumor regression in an isolated limb perfusion human melanoma xenograft model. Surgery 2002;132:252–8.

32. Skarsgard LD, Skwarchuk MW, Vinczan A, Kristl J, Chaplin DJ. The cytotoxicity of melphalan and its relationship to pH, hypoxia and drug uptake. Anticancer Res 1995;15:219–23.

33. Chaplin DJ, Acker B, Olive PL. Potentiation of the tumor cytotoxicity of melphalan by vasodilating drugs. Int J Radiat Oncol Biol Phys 1989;16:1131–5.

34. Economou JS, McBride WH, Essner R, et al. Tumour necrosis factor production by IL-2-activated macrophages *in vitro* and *in vivo*. Immunology 1989;67:514–9.

35. Maekawa H, Iwabuchi K, Nagaoka I, Watanabe H, Kamano T, Tsurumaru M. Activated peritoneal macrophages inhibit the proliferation of rat ascites hepatoma AH-130 cells via the production of tumor necrosis factor-α and nitric oxide. Inflamm Res 2000;49:541–7.

36. Albina JE, Reichner JS. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Rev 1998;17:39–53.

37. Bonnotte B, Larmonier N, Favre N, et al. Identification of tumor-infiltrating macrophages as the killers of tumor cells after immunization in a rat model system. J Immunol 2001;167:5077–83.