Combined Immunotherapy and Antiangiogenic Therapy of Cancer with Microencapsulated Cells

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ABSTRACT

An alternative form of gene therapy involves immunoisolation of a nonautologous cell line engineered to secrete a therapeutic product. Encapsulation of these cells in a biocompatible polymer serves to protect these allogeneic cells from host-versus-graft rejection while recombinant products and nutrients are able to pass by diffusion. This strategy was applied to the treatment of cancer with some success by delivering either interleukin 2 or angiostatin. However, as cancer is a complex, multifactorial disease, a multipronged approach is now being developed to attack tumorigenesis via multiple pathways in order to improve treatment efficacy. A combination of immunotherapy with angiostatic therapy was investigated by treating B16-F0/*neu* melanoma-bearing mice with intraperitoneally implanted, microencapsulated mouse myoblasts (C₂C₁₂) genetically modified to deliver angiostatin and an interleukin 2 fusion protein (sFvIL-2). The combination treatment resulted in improved survival, delayed tumor growth, and increased histological indices of antitumor activity (apoptosis and necrosis). In addition to improved efficacy, the combination treatment also ameliorated some of the undesirable side effects from the individual treatments that have led to the previous failure of the single treatments, for example, inflammatory response to IL-2 or vascular mimicry due to angiostatin. In conclusion, the combination of immuno- and antiangiogenic therapies delivered by immunoisolated cells was superior to individual treatments for antitumorigenesis activity, not only because of their known mechanisms of action but also because of unexpected protection against the adverse side effects of the single treatments. Thus, the concept of a “cocktail” strategy, with microencapsulation delivering multiple antitumor recombinant molecules to improve efficacy, is validated.

OVERVIEW SUMMARY

In this study, we have combined immunotherapy (via an interleukin 2 fusion protein) and antiangiogenic therapy (via angiostatin) against a B16-F0/*neu* mouse melanoma model. The proof-of-principle experiment had shown improved survival, with 30% of the animals surviving tumor free. Neutrophil-driven inflammation against capsules delivering immunotherapy was subdued by angiostatin, while tumor endothelial cell apoptosis driven by angiostatin was enhanced by interleukin 2. Thus, microencapsulation based on a combination strategy shows promise in delivering potent therapies for cancer treatment.

INTRODUCTION

IMMUNOISOLATION GENE THERAPY is a cell-based approach to deliver recombinant therapeutic molecules for the treatment of genetic and somatic diseases. In this approach, genetically modified nonautologous cells are isolated from the host's immune system via encapsulation within microcapsules fabricated from a biocompatible polymer. The microcapsules are designed to be permeable to the recombinant products and nutrients, but not to the host's immune mediators, which are excluded because of their larger size (Chang *et al.*, 1999). Efficacy has been demonstrated in several murine genetic models of human diseases such as dwarfism due to growth hormone deficiency

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(al Hendy *et al.*, 1995), lysosomal storage disease due to β -glucuronidase deficiency (Ross *et al.*, 2000a,b), and hemophilia B due to factor IX deficiency (Van Raamsdonk *et al.*, 2002). In addition to treating such monogenic Mendelian disorders, we wish to examine the potential of this technology in the treatment of somatic diseases such as cancer (Cirone *et al.*, 2001).

The application of microencapsulation as a drug delivery platform for the treatment of cancer has shown promising developments, as, for example, the delivery of angiostatin from microencapsulated cells to treat glioma in the central nervous system (Joki *et al.*, 2001; Read *et al.*, 1999). We have applied this technology to treat tumors by administering a HER-2/*neu*-targeted fusion protein to peripheral sites of an immunotherapy model. The fusion protein consisted of interleukin 2 (IL-2) fused to the variable region of a monoclonal antibody directed against a tumor surface antigen, the receptor for HER-2/*neu*. IL-2 is a 15-kDa immunostimulatory cytokine involved predominantly in T cell activation and proliferation (Gillis *et al.*, 1978) to stimulate the T cell-driven antitumor response. When this fusion protein (sFvIL-2) was delivered from genetically modified encapsulated cells, both tumor suppression and life span extension were achieved (Cirone *et al.*, 2002). However, long-term efficacy was limited by a gross neutrophil-driven inflammatory response generated by the biological activity of IL-2, leading to apoptosis of the encapsulated cells and only transient suppression of tumor growth. Hence, an alternative and cytokine-independent strategy was developed in which angiostatin was delivered to suppress tumor growth by inhibiting tumor angiogenesis.

The prominent role of angiogenesis has been well established in cancer progression and metastasis. An important inhibitor of angiogenesis, angiostatin, has been shown to inhibit neovascularization and metastasis (O'Reilly *et al.*, 1994). Its antiangiogenic (Cao *et al.*, 1996) and tumor-suppressing (MacDonald *et al.*, 1999) activities have been demonstrated in a variety of models *in vitro* (Cao *et al.*, 1996; Claesson-Welsh *et al.*, 1998) and *in vivo* (O'Reilly *et al.*, 1996; Cao *et al.*, 1998). When B16-F0/*neu* melanoma tumor-bearing mice were treated with encapsulated cells secreting angiostatin, tumor growth was drastically reduced and survival was improved (Cirone *et al.*, 2003). However, although tumor growth was arrested, angiostatin did not eliminate the tumor that was present in these animals. When these tumors were examined histologically, it was evident that although tumor vascular endothelial cells (ECs) were being eliminated as a result of the antiangiogenic therapy, the tumors maintained a form of atypical vasculature whereby the erythrocyte-tumor interface did not include ECs. This atypical vasculature was termed "vascular mimicry," describing the vascular channel observed (Cirone *et al.*, 2003) that may account for the escape from the angiostatin-induced suppression of tumor growth.

Thus, each of the above-described approaches was limited in its own way. Microcapsules delivering sFvIL-2 were the target of acute inflammation due to the prochemotactic nature of IL-2, leading to eventual cell death within the microcapsules, whereas tumors treated with encapsulated angiostatin-secreting cells eventually developed endothelial cell-independent forms of neovascularization. However, because angiostatin has been shown to be antichemotactic to neutrophils (Benelli *et al.*, 2002), we hypothesized that this may ameliorate the cytokine-induced inflammatory response. Hence, in a two-pronged strategy, the combined potency of immunotherapy and antiangio-

genesis may achieve improved tumor suppression and bypass the above-described undesirable side effects of the individual treatments. In the present study, this two-pronged strategy was examined by delivering sFvIL-2 (immunotherapy) and angiostatin (antiangiogenic therapy) concurrently via microencapsulated cells to evaluate their potential synergism in tumor suppression.

MATERIALS AND METHODS

Cell lines

Mammalian expression plasmid encoding sFvIL-2 (pcDNA-H520C9sFv-hIL-2) was constructed as described previously (Li *et al.*, 1999) by fusing sFv antibody cDNA (versus human HER-2) from baculovirus expression plasmid pAHCx-5209sFv to wild-type human IL-2 cDNA from plasmid pLW46. pcDNA-H520C9sFv-hIL-2 was transfected into a mouse myoblast cell line derived from CH3 mice, C₂C₁₂ (CRL-1772; American Type Culture Collection [ATCC], Manassas, VA), by calcium phosphate precipitation (Chang *et al.*, 1993), selected with G418, and screened by enzyme-linked immunosorbent assay (ELISA) for human IL-2 as described (Li *et al.*, 1999).

Mammalian expression plasmid pRC-CMV-AST (kindly provided by Y. Cao) (Cao *et al.*, 1998) encodes mouse kringle regions 1–4 of plasminogen fused to a hemagglutinin tag (HA) and driven by a cytomegalovirus (CMV) promoter. C₂C₁₂ cells were transfected with this plasmid, using ExGen 500 according to the manufacturer's guidelines (Fermentas, Burlington, ON, Canada). Clones were screened for secretion of HA-tagged angiostatin by endothelial cell viability assay (described below) and confirmed by Western blot.

B16-F0/*neu* mouse cells genetically modified from a B16-F0 parental cell line to express human HER-2/*neu* on the cell surface was a generous gift from L. Weiner (Fox Chase Cancer Center, Philadelphia, PA). These cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and G418 (400 μ g/ml). Human umbilical vascular endothelial cells (HUVECs) were obtained from the ATCC (CRL-1730) and maintained in M199 medium (Sigma, St. Louis, MO) supplemented with 20% FBS, 1% penicillin–streptomycin, and endothelial cell growth supplement (ECGS, 100 mg/ml; Sigma).

Angiostatin quantification by endothelial cell viability assay

Angiogenic activity was quantified by endothelial cell growth as described previously (Cao *et al.*, 1996, 1998; Grisicelli *et al.*, 1998; MacDonald *et al.*, 1999). Briefly, $1-3 \times 10^4$ HUVECs were plated into a 96-well plate in culture medium (M199 medium supplemented with 20% FBS, 1% penicillin–streptomycin, and ECGS [400 mg/ml]; Sigma) and incubated for 3 days with either medium from angiostatin-secreting cells, or serum, or tissue extract homogenized in 25 mM Tris, 0.1 M NaCl, 0.2% Triton X-100, pH 7.6 (homogenization buffer) at 1 ml of buffer per 100 mg wet weight of tissue. The viability of the HUVECs was then determined by alamarBlue assay (AccuMed, Westlake, OH). Rabbit angiostatin, a generous gift from M. Hatton (McMaster University, Hamilton, ON, Canada), was used as a standard.

Quantification of cytokines by ELISA

sFvIL-2 was quantified by ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Mouse serum samples were diluted to 3% final volume in the recommended reagent diluent supplemented with 50% FBS. Mouse liver and mesenteric tissues were similarly assayed (R&D Systems).

Animals and tumor model

Five- to 6-week-old C57BL/6 female mice (Charles River, Montreal, PQ, Canada) were used in accordance with Canadian Council on Animal Care guidelines. Serum from orbital bleeds or cardiac punctures was stored at -70°C until use.

The tumor model was developed by injecting 4×10^6 B16-F0/*neu* cells into the left flank of mice. Tumor dimensions were measured with calipers to estimate the volume according to $V = ab^2/2$, where a is the longest diameter and b is the shortest diameter (Heike *et al.*, 1997). Mice were killed with CO_2 once the tumor volume reached 1 cm^3 as per institutional guidelines for end-point criterion. Minimal tumor dose experiments were performed with this cell line to establish an experimentally acceptable tumor cell load per mouse (data not shown).

Microcapsule fabrication and implantation

Alginate–poly-L-lysine–alginate (APA) microcapsules containing either parental C_2C_{12} cells or the derivative cell lines (angiostatin-expressing cells [C_2C_{12} –AST] or sFvIL-2-expressing cells [C_2C_{12} –sFvIL-2]) were constructed as previously described (Van Raamsdonk and Chang, 2001). Briefly, cells were suspended in sterile-filtered 1.5% alginate (improved Kelmar; gift from NutraSweet Kelco, San Diego, CA) at a concentration of 2×10^6 cells/ml alginate solution. This was extruded through a concentric airflow into a 1.1% CaCl_2 bath, washed, and coated with poly-L-lysine (PLL) and alginate. The washes were performed as described (Van Raamsdonk and Chang, 2001) (each for 2 min unless otherwise noted) with successive CaCl_2 solutions in graded concentrations and buffers, coated with 0.05% PLL for 6 min, washed with 1.1% CaCl_2 and 0.9% NaCl, and coated 0.03% alginate (4 min). Capsules were visually inspected by phase-contrast microscopy to confirm their integrity and uniform diameters of $\sim 400 \text{ nm}$. The resulting microcapsules were used immediately for implantation. Three milliliters of capsules was implanted intraperitoneally into each mouse, using an 18-gauge catheter needle. This represents approximately 6230 ± 1010 capsules per mouse.

Viable cell number per capsule was determined by using alamarBlue (AccuMed) to measure the metabolic activity of encapsulated cells from a known number of microcapsules. For this purpose, capsules ($100 \mu\text{l}$) with $400 \mu\text{l}$ of growth medium were distributed to individual sterile 24-well plate wells. Fifty microliters of alamarBlue was added and incubated for 3–4 hr. A standard serial dilution of known numbers of C_2C_{12} cells in $500\text{-}\mu\text{l}$ aliquots (as determined by use of a Coulter Z_1 particle counter; Beckman Coulter, Mississauga, ON, Canada) was similarly processed. This metabolic activity was then compared with that generated from a standard dilution series containing known numbers of C_2C_{12} cells to yield cell number.

After the incubation, supernatants were removed for fluorometric analysis (excitation at 535 nm; emission at 590 nm). Flu-

orescence was calibrated against the cell number of the standard series to yield the cell number of the experimental samples. Capsules were then counted in each well; the value of which was then used with the viable cell number data to yield viable cell number per capsule.

Histological analysis

Tissues were fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) (Histology Laboratory, McMaster University Hospital, Hamilton, ON, Canada). Mitotic and apoptotic indices were obtained by counting the corresponding cells in 10 medium-power fields (MPFs, $\times 400$) per given slide (or over the entire area of the section in the case of smaller tumors). All such counts were performed in a blinded manner. This was repeated for five slides per animal (five mice per group). The indices were expressed as percent mitotic cells per total viable cells or apoptotic nuclei per total number of cells per MPF. Apoptosis measurements were validated with the TumorTACS *in situ* apoptosis detection kit (R&D Systems). The necrotic index was determined as the percentage of necrosis over the entire area of tumor.

Anti-von Willebrand factor immunohistochemistry

After paraffin-embedded sections were deparaffinized, antigen retrieval was performed with proteinase K treatment for 5 min followed by blocking of endogenous peroxidase activity by a 5-min treatment with 3% H_2O_2 (pharmacy grade). Primary antibody (rabbit anti-human von Willebrand factor [vWF]; DakoCytomation, Mississauga, ON, Canada) was added at a 1:500 dilution (diluted in 0.1 M phosphate buffer supplemented with 5% goat serum as a block) followed with a secondary antibody (Envision⁺, goat anti-rabbit IgG conjugated to peroxidase; DakoCytomation) and stained with liquid 3,3'-diaminobenzidine (DAB) substrate chromogen system (DakoCytomation) followed by counterstaining with hematoxylin (Sigma).

The degree of vascularization was assessed by comparing the area of vascularization with the area of the tumor. Areas were digitally quantified by image capture of the entire tumor section with Image-Pro 6.0 software (MediaCybernetics, Silver Spring, MD) and were compared with the known area of the slide's coverslip to attain area in units of square millimeters. Viable and nuclear condensed (apoptotic) endothelial cells were counted per sectional area of tumor. Values are reported as number of cells per sectional area of tumor. The total number of vWF-positive cells was converted in the entire tumor section to give ECs per tumor sectional area. The percentage of these vWF-positive cells showing nuclear condensation (apoptosis) was converted to give the EC apoptotic index. These values were then used to calculate the viable ECs per tumor sectional area. Specificity of anti-vWF staining for endothelial cells was confirmed in tumor sections in which the primary antibody was omitted. Angiostatin did not interfere with anti-vWF staining as shown by pretreatment of tumor sections with angiostatin (data not shown).

Cytotoxicity assay

For quantification of the cytotoxic T lymphocyte (CTL) response against B16-F0/*neu* tumor cells or C_2C_{12} mouse myo-

TABLE 1. EXPERIMENTAL PROTOCOL^a

Day	Injection/implantation	Experimental (COMBO)	Control I (angiostatin)	Control II (sFvIL-2)	Control III (mock) ^b
0	Tumor cells	+	+	+	+
3	Encapsulated cells–angiostatin	+	+	—	—
14	Encapsulated cells–sFvIL-2	+	—	+	—

^aMice were injected subcutaneously with tumor cells on day 0 and implanted with 3 ml of the respective microencapsulated cells intraperitoneally on day 3 and/or day 14. Four groups of mice (each group, $n = 10$) were treated as indicated.

^bMock control consisted of implanting encapsulated parental C₂C₁₂ cells on day 3.

blast cells, spleens were harvested from mice on day 21 after tumor cell injection. The splenocytes were then cocultured with the respective γ -irradiated target cells at an effector-to-target ratio of 30:1 for 5 days in RPMI medium (supplemented with 10% FBS, 1% penicillin–streptomycin, and 55 μ M culture-grade 2-mercaptoethanol). Live target cells (5×10^3) were then plated in 96-well culture plates to which the cultured effector cells were added in dilutions to yield effector-to-target ratios of 100:1, 50:1, and 10:1 in a total volume of 200 μ l per well. Cell death due to cytotoxicity was assessed by assaying the glucose 6-phosphate (G6P) released from terminated cells by use of a Vybrant cytotoxicity assay kit (Molecular Probes, Eugene, OR) after culturing these cells for 4 hr at 37°C. Experimental, maximal, and spontaneous releases of G6P were assessed as per the manufacturer's instructions. Final cytotoxicity was expressed as a percentage of the maximal response as described by the formula: % cytotoxicity = $100 \times [(\text{Abs}_{\text{Exp}} - \text{Abs}_{\text{Spontaneous}}) / (\text{Abs}_{\text{Max}} - \text{Abs}_{\text{Spontaneous}})]$.

RESULTS

Cell encapsulation, viability, and transgene expression

The design of the protocol is summarized in Table 1. In the combination (COMBO) group, encapsulated C₂C₁₂–angiostatin (C₂C₁₂–AST) cells were implanted into mice 3 days after injection of B16-F0/*neu* tumor cells and C₂C₁₂–sFvIL-2 cells, which secreted the immunoconjugate targeted to *neu*-expressing tumors, were implanted on day 14. These capsule implantations were chosen for their optimal efficacy for each treat-

ment alone (Cirone *et al.*, 2002, 2003). Three control groups of mice receiving either encapsulated C₂C₁₂–angiostatin cells or encapsulated C₂C₁₂–sFvIL-2 cells alone, or encapsulated but nontransfected C₂C₁₂ cells, were treated as shown in Table 1.

To assess the functional status of implants, we analyzed cell viability and transgene expression of the various groups of microcapsules before and after 21 days of implantation. Our previous work showed that capsules containing C₂C₁₂–sFvIL-2 cells became embedded in adhesions of fibrotic and necrotic tissue because of IL-2-driven inflammation (Cirone *et al.*, 2002). Thus, in mice implanted with IL-2-producing microcapsules, two populations of capsules were found on euthanasia on day 21: those within inflammatory adhesions and those free-floating within the peritoneal cavity. Only the free-floating microcapsules were recovered for these functional analyses (Table 2). It was clear that the cells within all recovered microcapsules had proliferated during the implantation period of 21 days, increasing from the preimplant level by 4- to 6-fold. However, whereas the angiostatin transgene expression level did not change significantly over the course of the experiment, immunoconjugate expression decreased to about 50% from preimplantation (Table 2). Interestingly, cells within capsules retrieved from the COMBO group were positive for angiostatin expression only, not for sFvIL-2 (Table 2). Furthermore, the level of angiostatin secretion in this COMBO group was not diminished, compared with the preimplantation level, even though there were capsules containing only sFvIL-2-secreting cells among these recovered capsules. The observed loss of sFvIL-2 expression could be due either to loss of viable sFvIL-2 cells or, alternatively, to silencing of sFvIL-2 expression. Hence, it is important to ascertain what types of capsule

TABLE 2. CELL VIABILITY AND TRANSGENE EXPRESSION OF ENCAPSULATED CELLS^a

	Control I (angiostatin)		Control II (sFvIL-2)		Experimental		
	Cells/capsule	Angiostatin (pg/cell)	Cells/capsule	sFvIL-2 (pg/cell)	Cells/capsule	Angiostatin (pg/cell)	sFvIL-2 (pg/cell)
Preimplantation	378 $\pm$ 82	2.34 $\pm$ 1.22	256 $\pm$ 42	0.206 $\pm$ 0.034	—	—	—
Retrieved	2510 $\pm$ 344 ^b	1.85 $\pm$ 0.80	968 $\pm$ 158 ^b	0.111 $\pm$ 0.004 ^c	1790 $\pm$ 164	2.29 $\pm$ 0.51	ND

^aEncapsulated cells were assayed for viability as well as angiostatin or sFvIL-2 expression before implantation and on retrieval from mice on day 21 after tumor cell injection. Transgene expression was determined in the medium collected after incubating the microencapsulated cells for 24 hr. Values were averaged from triplicate assays from $n = 3$ mice for the retrieved samples, and include the SEM.

^b $p < 0.05$.

^c $p < 0.01$.

FIG. 1. Immunohistochemical assessment of encapsulated cells in mice. Postmortem examination of the peritoneal cavities of mice on day 21 showed microcapsules either free in the peritoneal space (*free-floating*) or within inflammatory adhesions localized to lymphoid tissue of the mesentery and spleen (*adhesion*). Immunohistochemical staining against the HA tag of the recombinant angiostatin [(F–J) *anti-HA*; red] or against the human IL-2 component of sFvIL-2 [(K–O) *anti-hIL-2*; green] was performed on these two types of microcapsule. Free-floating capsules were dissolved with citrate to release the cells onto slides. Adhesion samples were from paraffin-embedded mesenteric tissue sections. In the free-floating microcapsules, only recombinant angiostatin was detected. No cells expressing sFvIL-2 were found among these samples. In the microcapsules imbedded within adhesions, only sFvIL-2-expressing cells were detected; none of the cells within the microcapsules expressed angiostatin. However, neutrophils surrounding these capsules were positive for anti-HA stain (I; arrow). Arrowheads indicate microcapsules. Scale bar: 50 μ m (*free-floating*); 100 μ m (*adhesion*).

were included among these recovered free-floating microcapsules and what types were embedded in the inflammatory adhesions.

To resolve these issues, we analyzed these two types of microcapsule for IL-2 and angiostatin expression by immunohistochemistry (Fig. 1). All capsules within adhesions from the COMBO treatment group stained positively for sFvIL-2 (Fig. 1N), similar to those from the control group with sFvIL-2 treatment alone (Fig. 1L). However, recombinant angiostatin was not detected in these capsules from the COMBO group (Fig. 1I), but interestingly, it was associated with the surrounding neutrophils instead (arrow in Fig. 1I). In the control group with angiostatin treatment alone, capsules were seldom found within adhesions. In the rare instance in which a capsule was found (Fig. 1H), recombinant angiostatin was detected in the enclosed cell clump, as expected. Immunohistochemical analysis of cells within freely floating capsules from the COMBO group showed rather the opposite results. Recovered cells consisted entirely of angiostatin-positive cells (Fig. 1J), whereas IL-2-positive cells were absent (Fig. 1O).

Efficacy of immunotherapy combined with antiangiogenic therapy

The essential proof of principle that combining immunotherapy with antiangiogenic therapy can yield an improved antitumor response was shown in two ways: delayed tumor growth (Fig. 2A) and improved survival (Fig. 2B). Tumors receiving any of the three treatments (COMBO, angiostatin, or sFvIL-2) were smaller than those of mock controls by day 21. The COMBO group displayed the least tumor growth and the most improved survival. Importantly, 20% of COMBO-treated mice were found to be tumor free and 30% of these mice survived beyond day 50 after tumor injection. Therefore, this double-pronged approach was shown to be a superior treatment compared with that of individual therapy alone.

Systemic delivery of therapeutic products

To test whether the distribution of either angiostatin or sFvIL-2 to the systemic circulation was affected by COMBO

FIG. 2. Efficacy of antiangiogenic therapy in combination with immunotherapy. Four groups of mice (each group, $n = 10$) were injected with B16-F0/*neu* tumor cells as detailed in Table 1. (A) Tumor volumes of subcutaneous B16-F0/*neu* tumors were measured up to day 21. (B) Kaplan-Meier plot of animal survival (end point defined by tumor volume reaching 1 cm³). Significant differences were obtained on day 21 between the experimental group and the three control groups: $**p < 0.01$; $***p < 0.001$. Error bars represent the SEM.

FIG. 3. Systemic delivery of sFvIL-2 and angiostatin. sFvIL-2 (A) and angiostatin (B) in serum were determined on day 21 after tumor cell injection in the four groups as detailed in Table 1. Similar concentrations of sFvIL-2 were detected in the experimental group and the control group receiving encapsulated C₂C₁₂-sFvIL-2 cells alone. Angiostatin was detected in mice receiving encapsulated C₂C₁₂-AST cells either alone or in the COMBO group. Significant differences are relative to mock control unless otherwise indicated: * $p < 0.05$; ** $p < 0.01$.

treatment, we assayed for sFvIL-2 and angiostatin in serum, tumors, and peritoneal organs. Day 21 was chosen as the appropriate sampling time because, as noted previously (Cirone *et al.*, 2003), angiostatin was localized to the tumor until around this time, concurrent with the loss of endothelial tumor cells.

Recombinant products in sera on day 21 were found in the COMBO group of mice at levels comparable to those in mice treated with the single therapies (Fig. 3). The immunoconjugate sFvIL-2 concentration, despite being much less than that of angiostatin, was still measurable in similar amounts (~1.5 ng/ml serum) in both immunotherapy-treated mice and COMBO-treated animals. As expected, mock-treated and angiostatin-

treated groups showed no detectable level of sFvIL-2 in serum (Fig. 3A). This confirmed that mice in the COMBO group received the same level of immunoconjugate as with immunotherapy alone. Although the level of angiostatin in serum was also comparable between the COMBO group and the angiostatin-treated group, there was a slightly decreased amount in the COMBO group, but the difference was not statistically significant. Again, no recombinant angiostatin was detected in the mock-treated group or the sFvIL-2-treated group, as expected (Fig. 3B).

The distribution of the two recombinant products in various body organs and tumors was markedly different (Fig. 4). For

FIG. 4. sFvIL-2 and angiostatin delivery to organs and tumors. Tumor and organ extracts were assayed on day 21 for angiostatin (A) and sFvIL-2 (B) in the four groups of mice as detailed in Table 1. $n = 5$ mice per group except in the experimental group, with $n = 3$. Values represent mean values and error bars represent the SEM. Significant differences are relative to mock controls unless otherwise indicated: * $p < 0.05$; ** $p < 0.01$.

angiostatin, the highest and indeed the most physiologically relevant accumulation was in the tumor tissue. This was true for both the angiostatin-alone and COMBO-treated animals (Fig. 4A). The rest of the organs (kidney, liver, and spleen) showed negligible amounts of angiostatin. This pattern of distribution was markedly different from that of sFvIL-2. Instead of accumulating in the tumor tissue, recombinant sFvIL-2 was detected mainly in the kidney, liver, and spleen of mice treated with sFvIL-2 alone. In the COMBO group, while the kidney and liver showed levels similar to those of the sFvIL-2 single-treatment group, the spleen showed a dramatically decreased and negligible amount of sFvIL-2, similar to that of the mock and angiostatin-treated groups (Fig. 4B).

Histological assessment of peritoneal organs and tumors from treated mice

A histological assessment of relevant tissues was done to examine the effects of COMBO treatment (Fig. 5). Both liver and kidney tissues appeared normal, with no difference among the

treatment groups (alone or in combination), or relative to the mock control (data not shown). Changes in spleen, mesenteric tissue, and mesenteric lymph nodes, however, were noted (representative sections in Fig. 5).

Among the mock-treated animals, mild reactive changes were observed in some spleens, characterized by a mild increase in the mixture of inflammatory cells including scattered megakaryocytes (Fig. 5A, panel i). Single cell-layered mesothelium in the mesentery also showed a mild reaction caused by contact with the capsules (Fig. 5B, panel i), but this did not affect viability of encapsulated unmodified C₂C₁₂ cells. Lymph node and tumor histology was unremarkable.

Among the angiostatin-treated mice, the white pulp of the spleen displayed minor changes with a possible increase in the number of follicular centers. However, the main change was in the red pulp region, with an increase in extramedullary hematopoiesis that included a significant population of megakaryocytes as compared with the mock-treated animals (Fig. 5A, panel ii). The lymph nodes showed some reactive changes including follicular hyperplasia, paracortical expansion, and si-

FIG. 5. Tissue histology. Spleen (A), mesentery (including capsules whenever possible) (B), lymph nodes (C), and tumors (D) from each of the four groups of mice obtained postmortem on day 21 were processed for H&E staining. Representative fields of the tissue sections are presented. Scale bars: 500 μ m (spleen, lymph node, and tumor); 100 μ m (mesentery). M, mesothelial layer of cells in mesentery; C, sample capsules; S, areas of sinusoidal dilation in lymph nodes.

nus histiocytosis as well as a dilation of the sinusoid (Fig. 5C, panel ii). The changes were slightly more evident than those noted in the mock-treated lymph nodes.

Among the sFvIL-2-treated animals, mesenteric sections

showed several embedded capsules within an inflammatory exudate, consisting of cellular debris with a mixed inflammatory infiltrate that included chronic inflammatory cells as well as several neutrophils that immediately surrounded the capsules

TABLE 3. HISTOLOGICAL ANALYSIS OF TUMOR TISSUES^a

	Mitotic index (%)	Apoptotic index (%)	Necrotic index (%)
Untreated	1.50 $\pm$ 0.16	2.48 $\pm$ 0.35	34 $\pm$ 5
sFvIL-2	1.43 $\pm$ 0.35	4.09 $\pm$ 0.76 ^{b,d}	57 $\pm$ 7 ^{b,f}
Angiostatin	1.38 $\pm$ 0.11	5.12 $\pm$ 0.47 ^{b,e}	47 $\pm$ 6 ^{c,f}
COMBO	1.57 $\pm$ 0.27	8.91 $\pm$ 0.75 ^c	64 $\pm$ 4 ^c

^aH&E-stained tumor sections from the four groups of mice (as described in Table 1 except for the untreated controls, which did not receive any microcapsules) were analyzed morphometrically to yield mitotic, apoptotic, and necrotic indices. $n = 5$ mice per group except for the experimental group with $n = 3$ mice.

^b $p < 0.01$, relative to the Untreated group.

^c $p < 0.001$, relative to the Untreated group.

^d $p < 0.05$, relative to the COMBO group.

^e $p < 0.01$, relative to the COMBO group.

^f $p < 0.001$, relative to the COMBO group.

(Fig. 5B, panel iii). In the spleen (Fig. 5A, panel iii) and lymph nodes (Fig. 5C, panel iii), there was dramatic follicular hyperplasia. Sections of sFvIL-2-treated spleens showed a more dramatic increase in extramedullary hematopoiesis within the red pulp than that observed in the angiostatin-treated group. Lymphoid follicles within the white pulp appeared to have increased in number. Within the lymph nodes, several reactive changes were observed. These changes included follicular hyperplasia, expansion of the paracortical region, as well as significant sinusoidal dilation and sinus histiocytosis. In general, several indicators of immune activation were present including well-formed germinal centers in the follicles and expansion of the paracortical region (Fig. 5C, panel iii).

Among the COMBO-treated animals, the spleens showed follicular hyperplasia, as noted in the sFvIL-2 group. This was not found in the angiostatin- and mock-treated animals (data not shown). Furthermore, splenomegaly was observed in 70% of COMBO-treated animals and 100% of animals receiving the IL-2 fusion protein alone (Fig. 5A, panel iii). In contrast, all of the angiostatin-treated (Fig. 5A, panel ii) and mock-treated (Fig. 5A, panel i) animals, as well as 30% of the COMBO-treated animals (Fig. 5A, panel iv), had normal size spleens. In the COMBO group, there was also a dramatic reactive inflammatory response within the mesenteric fat with embedded capsules (Fig. 5B, panel iv). However, there was less necrotic cellular debris than in the sFvIL-2-treated animals (Fig. 5B, panel iii). It is possible that the neutrophil-driven response was suppressed in these animals, consistent with the notion of the antichemotactic effect of angiostatin on neutrophils.

Morphometric assessment of tumors from treated mice

Tumors from the four groups of mice (Fig. 5D) were used for detailed analysis for mitosis, necrosis, and apoptosis (Table 3). Neither single nor combination treatments had a major effect on the mitotic index of B16-F0/*neu* tumors (Table 3, mitotic index). Necrotic and apoptotic indices both increased in all treatment groups and significantly more so in the COMBO group (Table 3). The necrosis observed with the combination treatment, although greater than with either of the two single treatments, was clearly neither additive nor synergistic (Table 3). However, tumor apoptosis observed in the COMBO group appeared to be additive from the individual treatments (Table 3).

The extent of antiangiogenic effect on tumor endothelial cells (ECs) was examined with anti-von Willebrand factor (vWF, an endothelial cell/platelet-specific marker) staining and then evaluated morphometrically for EC content, apoptosis, and viability (Fig. 6). As sFvIL-2 was not known to affect angiogenesis, all three EC morphometric analyses showed no difference in the sFvIL-2 single-treatment group compared with the untreated or mock controls in any of the above-described parameters.

As antiangiogenic activity would kill off endothelial cells, the number of endothelial cells in treated tumors should decrease. This was confirmed in the angiostatin single-treatment group (Fig. 6). The EC indices for tumors treated with angiostatin alone showed dramatic changes from the mock controls, showing a highly significant decrease in EC density and an increase in EC apoptosis (Fig. 6). Interestingly, in the COMBO-treated tumor sections, there was an apparent dramatic increase in EC content per tumor sectional area, but this increase was associated with an increase in EC apoptosis and a decrease in viable ECs per

tumor section in the COMBO group (Fig. 6). Hence, the apparent increase in ECs per tumor section is likely due to the decrease in tumor volume (Fig. 2A), whereas the increase in efficacy of the COMBO group was associated in part with increased apoptosis of the ECs (Fig. 6). There was a slightly increased number of viable ECs per tumor sectional area for the COMBO treatment group compared with the angiostatin group, but the difference was not statistically significant (Fig. 6).

Vascular mimicry in COMBO-treated tumors

Interestingly, when tumor sections were stained for vWF, some tumor vasculature was noted to exist in the absence of

FIG. 6. Histological analysis of endothelial cells in tumor tissue. Tumor sections from the four groups of mice (Table 1) were stained for vWF-positive endothelial cells (ECs) and underwent morphometric analysis as described in Materials and Methods. The number of vWF-positive cells per tumor sectional area was determined (A) and an assessment of apoptotic vWF-positive cells was performed (B). Data indicating total cell number and proportion of those undergoing apoptosis were thereafter used to yield viable EC counts per tumor sectional area (C). $n = 3$ mice per group; error bars represent the SEM. * $p < 0.05$; ** $p < 0.01$ compared with mock controls unless otherwise indicated.

endothelial cells (Cirone *et al.*, 2003). Such channels were characterized by an erythrocyte–tumor interface without an endothelial cell lining around the vessel. Vessels were noted to have either a continuous layer of endothelial cells, a discontinuous layer of endothelial cells, or no endothelial cells at all. Tumor sections stained immunohistochemically for vWF were analyzed morphometrically (Fig. 7). Tumors for each of the four groups of animals were assessed for the relative contributions of vessels with a continuous layer of endothelial cells (all ECs) and those that lacked endothelial cells altogether (no ECs). The specificity of anti-vWF staining was verified to show that this was not an artifact due to interference by angiostatin (see Materials and Methods). Treatment with angiostatin significantly decreased the prevalence of vasculature with a continuous layer of ECs (all ECs), while concurrently raising the prevalence of vasculature without any endothelial cells (no ECs) (Fig. 7). This was true for the groups receiving angiostatin alone or the COMBO treatment. IL-2 immunotherapy alone did not affect vascular profiles when compared with the mock control. However, when it was combined with angiostatin in the COMBO group, it led to an even greater increase in the proportion of “no EC” vasculature.

T cell recruitment to tumors and CTL activation

Because T cell recruitment may be affected by angiostatin, anti-CD3⁺ cell staining of tumor sections was performed for each group of treated mice. The specificity of the stain was demonstrated in a normal lymph node (Fig. 8A and B) as a positive control and in tumor tissues (Fig. 8C and D). The CD3⁺ cell counts per field of view showed that both sFvIL-2 and angiostatin single treatments yielded increased recruitment of T cells to the tumor compared with the mock control (Fig. 8E). As sFvIL-2 is known to stimulate lymphocyte proliferation, the increase in CD3⁺ cell counts observed in sFvIL-2-treated tu-

mors was expected. A similar increase in CD3⁺ cell counts in angiostatin-treated tumors was consistent with angiostatin having some role in leukocyte trafficking/recruitment. However, the COMBO treatment groups yielded the highest level of CD3⁺ cells per tumor sectional area (Fig. 8E), leading to a 3-fold increase compared with the mock control.

Angiostatin currently has no known direct effect on immune function. As angiostatin alone or in combination with immunotherapy showed increased T cell recruitment to the tumor (Fig. 8), it was necessary to assess whether angiostatin alone could indeed activate lymphocytes. This was assessed by a cytotoxic T lymphocyte (CTL) assay against B16-F0/*neu* tumors. In mice receiving sFvIL-2 treatment alone, or COMBO treatment, a strong and similar activation of T cells was observed against target tumor cells in culture, as expected (Fig. 9). However, angiostatin treatment alone did not alter T cell activation, compared with the mock control. Therefore, the increased recruitment of lymphocytes in the angiostatin-treated tumor (Fig. 8E) was not the result of activation of T cells by the treatment.

DISCUSSION

The increased efficacy of the COMBO treatment over the angiostatin or sFvIL-2 single treatment was shown clearly in Fig. 2, whereby the combination treatment delayed tumor growth and improved survival. In addition, 30% of COMBO-treated mice were tumor free by day 50, well beyond the end point for the mock control on day 21. None of the mice in the other treatment groups was ever tumor free and all required euthanization because of the presence of a large tumor. Several mechanistic advantages of the combined approach contributed to this outcome. First, the improved efficacy was concurrent with an increase in tumor apoptosis and necrosis (Table 3)—

FIG. 7. Vascular mimicry. Tumor sections from the four groups of mice (Table 1) were stained for vWF, and tumor vasculature was sorted according to endothelial cell content surrounding the observed vessel. Vasculature observed to consist of a continuous layer of endothelial cells (vWF positive) was categorized as the *All EC* group. Vessels consisting of an erythrocyte–tumor interface with no vWF-positive cell layer were in the *No EC* group. Vasculature consisting of an intermediate type with a discontinuous layer of vWF-positive cells was also analyzed (data not shown). Entire tumor sections were examined under several medium-power fields of view and the surface area of the vasculature relative to the tumor sections was assessed by morphometric analysis, using Image-Pro 6.0 software (see Materials and Methods). $n = 5$ mice per group (five sections per mouse), except for the COMBO group ($n = 3$). Error bars represent the SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with mock controls unless otherwise indicated.

FIG. 8. T cell recruitment to tumors. Immunohistochemical staining for CD3 (a T cell marker) was used to analyze the recruitment of T cells to the lymph nodes of the various groups of mice. Two-micron serial sections of the paracortical region of the lymph nodes from an untreated mouse (A and B) and tumor from a COMBO-treated mouse (C and D) were used to test the specificity of the stain. (A and C) Negative controls lacking primary antibodies to CD3. (B and D) Normal staining for CD3. (E) Tumor sections from the four groups of mice (see Table 1) for immunohistochemical analysis of CD3-positive cells. FOV, Field of view (at $\times 100$ magnification) of the tumor sections. Scale bar: 100 μm ; 500 μm for insets. $n = 5$ mice per group (five sections per mouse), save for the COMBO group ($n = 3$). Error bars represent the SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with mock controls unless otherwise indicated.

both direct indices of tumor death and tumor endothelial cell apoptosis (Fig. 6). Angiostatin and sFvIL-2 also did not impede each other's delivery systemically (Fig. 3) or to other tissues except in the spleen, where the expected high level if sFvIL-2 was not observed in the COMBO group (Fig. 4). The COMBO-treated mice also had fewer cases of enlarged spleens than those receiving only immunotherapy. Hence, concurrent angiostatin treatment provided an additional benefit by reducing the incidence of splenomegaly caused by IL-2. This may have been mediated through increased clearance of IL-2 by the sinusoidal dilation observed in the lymph nodes of the COMBO-treated animals (Fig. 5A and C), a consequence that can be attributed to angiostatin treatment.

IL-2 is known to be proinflammatory and has chemotactic activity toward neutrophils. IL-2 can directly stimulate and increase neutrophil adherence to endothelial cells *in vitro* by enhancing the expression of CD18 and possibly other adhesion molecules on the surface of neutrophils (Li *et al.*, 1996). This has been proposed as a cause for the vascular leak syndrome by immunotherapy with bolus IL-2. As angiostatin had been shown to be able to inhibit neutrophil chemotaxis *in vitro* (Benelli *et al.*, 2002), we hypothesized that the addition of an-

giostatin could inhibit neutrophil-driven inflammation of the encapsulated sFvIL-2 cells. This could be seen in H&E-stained tumor sections or mesenteric tissues local to the capsules (Fig. 5). Several necrotic areas were noted around the capsules from sFvIL-2-treated animals but clearly less necrosis was observed in similar sections from combination-treated animals, and none was observed in the angiostatin-treated group. This indicates that the inflammatory response did not progress in the COMBO group as readily as in the sFvIL-2 group.

The loss of IL-2 expression has been a persistent problem in gene therapy protocols. Whether by viral vectors (Addison *et al.*, 1995; Toloza *et al.*, 1996) or *ex vivo* methods (Cao *et al.*, 1999a,b; He *et al.*, 2000), none have been able to deliver biologically active IL-2 beyond a few weeks. When sFvIL-2 was delivered via immunisolated microencapsulated cells, a pervasive neutrophil-driven inflammatory response was still observed (Cirone *et al.*, 2002), leading to the elimination of encapsulated cells that expressed the cytokine (Table 2) and neutrophil accumulation around the capsules (Fig. 1). Hence, because angiostatin can inhibit neutrophil chemotaxis (Benelli *et al.*, 2002), either directly or through interaction with $\alpha_v\beta_3$ -integrin on endothelial cells, the concurrent delivery of angio-

FIG. 9. Cytotoxic T lymphocytes against B16-F0/*neu* tumor cells. CTL activity is significantly activated in the presence of sFvIL-2 either alone or in combination with angiostatin (COMBO). Conversely, angiostatin treatment alone did not activate CTLs over the mock controls or enhance the response in the COMBO group.

statin in the present COMBO treatment is able to ameliorate this cytokine-induced inflammatory response. However, although this effect was observed to some extent, it was not sufficient to eliminate this reaction entirely (Figs. 1 and 5).

Another important result was the high level of angiostatin observed at the tumor site (Fig. 4). As the tumor is one of the exclusive sites for active angiogenesis in the adult animal, it provides a target-rich environment for angiostatin. Indeed, when taking into account the amount of angiostatin found at the tumor and generated from the microcapsules in the mouse (with an angiostatin half-life of approximately 2.5 days [Hatton *et al.*, 2001; Cirone *et al.*, 2003]), nearly all the angiostatin appeared to be localized to the tumor. Thus, angiostatin is potentially one of the best tumor-targeting moieties available. Therefore, a strategy of fusing angiostatin to other cancer drugs may be a potent anticancer therapy to be considered.

It is clear that increased necrosis and apoptosis accounted for much of the efficacy of the combination treatment against tumors (Table 3). The necrosis observed (Table 3) also indicated an overlap of efficacy. IL-2 is known to stimulate cytotoxic T lymphocytes to direct tumor killing while angiostatin drives endothelial cell apoptosis. Hence, the tumor toxicity is likely due to a cumulative effect of these independent mechanisms. Because angiostatin was unable to alter cell-mediated cytotoxicity against tumor cells (Fig. 9), it likely does not have a direct effect on T cells. Instead, its apoptotic effect on endothelial cells may promote better T cell access and recruitment to tumors, hence accounting for enhanced T cell localization to tumors (Fig. 8).

As sFvIL-2 has no direct effect on tumor angiogenesis, the increase in endothelial cell apoptosis with COMBO treatment (Fig. 6) likely comes from some secondary effect. It has been shown that tumor endothelial cells can provide antigens distinct

from other endothelial cells and can subsequently be used to vaccinate against tumors (Niethammer *et al.*, 2002). Hence, for the COMBO-treated animals, which had received angiostatin since day 3, there would be time to allow antigen-presenting cells to present tumor endothelial cell antigens to T cells, thus accounting for the increased EC apoptosis observed in the COMBO-treated animals and leading to the greater efficacy of COMBO treatment (Fig. 2).

It has been shown that some highly metastatic breast and melanoma cancers develop a vascular network despite the lack of classic blood vessels (Maniotis *et al.*, 1999), a phenomenon described as "vasculogenic mimicry." This was also observed in our angiostatin-treated animals, which showed an increased prevalence of vWF-negative erythrocyte-tumor interfaces (Fig. 6). We described this phenomenon as "vascular mimicry," to indicate that they are the result of treatment, as opposed to there being a direct genetic cause for this atypical vasculature. This mechanism may have accounted for the eventual escape of tumor from the antiangiogenic therapy. However, the addition of sFvIL-2 immunotherapy may have helped to overcome tumor escape by vascular mimicry, as some mice receiving the COMBO treatment were tumor free and did not succumb to continued tumor cell proliferation (Fig. 2).

In combination with angiostatin, sFvIL-2 does contribute to the antiangiogenic effect as noted by the significantly increased tumor EC apoptosis and the increase in no-EC channels (i.e., vessels consisting of an erythrocyte-tumor interface with no vWF-positive cell layer) compared with angiostatin-alone treatment (Fig. 7). It is thus interesting to consider the effect of vascular mimicry on leukocyte diapedesis. Clearly, if there were no EC barrier between blood and tumor, tumor cells would be readily exposed to leukocytes, resulting in more efficacious tumor cell killing by the IL-2-induced T cell response. Thus, al-

though sFvIL-2 does not affect tumor endothelial cells directly, but does in combination with angiostatin, it may overcome tumor escape by vascular mimicry via an improved antitumor response, in addition to breaking anergy to tumor endothelial cells.

In conclusion, COMBO treatment with both IL-2 and angiostatin delivered from encapsulated cells was superior to single treatment in suppressing tumor growth and prolonging survival. This increase in efficacy was due not only to the accumulated effects of targeting multiple pathways in tumorigenesis (Cirone *et al.*, 2001), but also to secondary effects in which undesirable reactions caused by each reagent singly (IL-2-induced inflammation, angiostatin-induced vascular mimicry) were inadvertently ameliorated. Thus, the use of combination therapy in the treatment of multifactorial disorders such as cancer is of potential clinical importance.

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