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A MULTI-PRONG APPROACH TO CANCER GENE THERAPY BY CO-ENCAPSULATED CELLS

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ABSTRACT

Microencapsulation of non-autologous cells allows the same recombinant therapeutic cell line to be implanted in different recipients. This approach was successful in treating HER2/neu-expressing tumors in mice by delivering an interleukin-2 fusion protein (sFvIL-2), or angiostatin. However, inflammatory response to interleukin-2 and endothelial cell-independent neovascularization (vascular mimicry) with angiostatin treatment prevented the achievement of long-term efficacy. We hypothesized that co-encapsulating the two producer cells in the same microcapsules may enhance the efficacy and ameliorate the above side-effects. Hence, B16-F0/neu tumor-bearing mice were implanted with sFvIL-2- and angiostatin-secreting cells co-encapsulated in the same alginate-poly-L-lysine-alginate microcapsules. However, only an incremental and not synergistic improvement was achieved. Compared to the single sFvIL-2 treatment, there was improved efficacy associated with: mobilization of sFvIL-2 from the spleen; higher level of cytokine delivery systemically and to the tumors; increased tumor and tumor-associated endothelial cell apoptosis; and reduced inflammatory response. However, compared to the single angiostatin-treatment, the efficacy was reduced, primarily due to a “bystander” effect in which the angiostatin-secreting cells suffered similar transgene silencing as the co-encapsulated cytokine-secreting cells, but “vascular mimicry” was reduced. Hence, while there was no synergy in efficacy, an incremental improvement and significant reduction in undesirable side effects were achieved. (200 words)
INTRODUCTION

The implantation of genetically-modified non-autologous cells in immunoprotected microcapsules is an alternative to ex vivo gene therapy. Such cells delivering a recombinant therapeutic product are isolated from the host’s immune system by being encapsulated within biocompatible microcapsules. This approach has been successful in pre-clinical animal studies involving delivery of hormone or enzymes to treat dwarfism\(^1\), lysosomal storage disease\(^2,3\) or hemophilia B\(^4\). By extension, there is a significant clinical interest to determine how this technology may provide treatment for more complex diseases such as cancer.

The use of this immuno-protective technology in anti-cancer applications began in the late nineties. The pioneering work involved the encapsulation of cells expressing cytochrome P450 enzymes for converting a benign prodrug (ifosfamide) into a toxic metabolite for the treatment of pancreatic cancer\(^5,6\). Such encapsulated cells implanted close to the tumor site provided an alternate source of cytochrome P450 activity (the liver being the primary source). This strategy was to overcome limitations such as high toxicity and systemic dilution experienced with the bolus injection of the prodrug\(^7,9\). We have used a more direct approach for the treatment of cancer by delivering interleukin-2 (IL-2) to stimulate the immune rejection of tumor cells\(^10\). An IL-2 fusion protein targeted to the HER2/neu receptor on tumor cells was delivered from genetically modified encapsulated cells to treat murine solid tumors overexpressing the HER2/neu receptor\(^10\). However, efficacy was limited by a gross neutrophil-driven inflammatory response to the IL-2 biological activity, leading to death of the encapsulated cells and cessation of cytokine delivery. Hence, although efficacious in suppressing tumor growth initially, this immune therapy protocol only produced a transient effect, with the tumors resuming growth after a delay of ~2 weeks.
In addition to the above immunotherapeutic approach, anti-tumor angiogenesis (the propagation of blood vessels in tumors required for tumor growth and metastasis\textsuperscript{11}), was also targeted as a potential cancer treatment. The prominent role of angiogenesis has been well established in cancer progression and metastasis. Angiostatin is an internal, N-terminal fragment proteolytically cleaved from plasminogen\textsuperscript{12} with tumor-suppressing activity\textsuperscript{13}. It specifically reduces endothelial cell proliferation, migration, invasion and tube formation\textsuperscript{14,15,15-19} while the recombinant form inhibits primary angiogenesis-dependent tumor growth and metastases in mice\textsuperscript{20-27}. The microencapsulation technology has been used previously to deliver endostatin, another angiogenesis inhibitor, and was shown effective in suppressing gliomas in the murine CNS\textsuperscript{28,29}. In our laboratory, we had successfully delivered angiostatin systematically by implanted encapsulated recombinant cells in the peritoneal cavity to treat a B16-F0/neu mouse melanoma, leading to suppression of tumor growth and extension of life span\textsuperscript{30}. However, long-term efficacy was not achieved as the tumors eventually recurred. This was attributed in part to a phenomenon of vascular mimicry\textsuperscript{30}, in which neo-vascularization of the tumors was established without the participation of von-Willebrand-positive endothelial cells. We now propose to use a “co-encapsulation” approach in which both the IL-2 fusion protein- and angiostatin-secreting cells were encapsulated together for implantation. Through this two-prong approach, it may be possible to improve efficacy by reducing the intensity of the IL-2 induced inflammatory response and the angiostatin-induced vascular mimicry.
METHODS AND MATERIALS

Cell lines

The CH3-mouse strain derived C2C12 cells (ATCC, Rockville, Md; catalogue #CRL-1772) were transfected with plasmid expression vectors that encoded either sFvIL-2 or angiostatin (K1-4) and screened according to methods described\textsuperscript{10,30}.

B16-F0/neu mouse cells genetically modified from a B16-F0 parental cell line to express human HER-2/neu on its cell surface was a generous gift from Dr. Louis Weiner, Fox Chase Cancer Center, Philadelphia, PA. The B16-F0/neu were maintained in Delbecco’s minimal media supplemented with 10% FBS, 1% penicillin / streptomycin and 1.2 mg/mL of G418.

Human umbilical vascular endothelial cells were obtained from the ATCC (Rockville, MD; catalogue #CRL-1730) and maintained in M199 media (Sigma, Mississauga, Ontario) supplemented with 20% FBS, 1% penicillin / streptomycin and 100mg/ml of endothelial cell growth supplement (ECGS) (Sigma, Mississauga, Ontario).

Quantification of Angiostatin by Endothelial Cell Viability Assay

The angiogenic activity was quantified by endothelial cell growth as described previously\textsuperscript{30}. Briefly, 1-3 x 10\textsuperscript{4} HUVEC cells were plated into a 96-well plate in culture media (M199 media supplemented with 20% FBS, 1% P/S and 400mg/ml of Endothelial Cell Growth Factor Supplement (Sigma, Mississauga, Ontario) and incubated for 3 days with either media from angiostatin-secreting cells, or serum, or tissue extract homogenized in a 25mM Tris, 0.1M NaCl, 0.2% TritonX-100, pH 7.6 (homogenisation buffer) at 1ml buffer per 100mg of wet weight tissue. The viability of the HUVEC cells was determined with the alamarBlue assay (AccuMed, Westlake, OH) as per the supplier’s instructions. Rabbit angiostatin, a generous gift from Dr. Mark Hatton (McMaster University, Hamilton, Ontario), was used as a standard.
Quantification of sFvIL-2 ELISA

sFvIL-2 was quantified via an 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.

Animals and Tumor Model

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

The tumor model was developed by injecting $4 \times 10^6$ B16-F0/neu cells into the left flank of mice. Tumor dimensions were measured with calipers to estimate the volume according to $V = \frac{a b^2}{2}$, where $a$ was the longest diameter and $b$ the shortest. Mice were euthanized with CO$_2$ once the tumor volume reached 1cm$^3$ 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).

Microencapsulation

Alginate-Poly-L-Lysine-Alginate (APA) microcapsules containing cells were constructed as previously described. Briefly, cells were resuspended in sterile-filtered 1.5 % alginate (Improved Kelmar, a gift from the NutraSweet Kelco Company, San Diego, CA) at a concentration of 2 million cells per mL alginate solution, followed by a series of washes whereby coatings of poly-L-lysine and alginate were added. The resulting APA microencapsulated cells were used immediately for implantation. Capsules were visually inspected to confirm their integrity and uniform diameters of ~400nm. To co-encapsulate two cell types, both cells were
mixed in alginate together prior to the extrusion of the alginate-mix through a needle to form the microcapsules.

**Histological Analysis**

Tissues were fixed and processed for paraffin sectioning and Hematoxylin and Eosin (H&E) staining by standard protocols (Histology Laboratory, McMaster University Hospital, Hamilton, Ontario). Mitotic and apoptotic indices were obtained by counting the corresponding cells in 10 high power fields (HPF) (400X) per given slide (or over the entire area of the section in the case of smaller tumors). This was repeated for five slides per animal (5 mice per group). When the tissue area was less than 10 HPFs, the entire area was included. These indices were expressed as percent mitotic cells per total viable cells (per HPF) and apoptotic nuclei per total number of cells (per HPF). Apoptosis measurements were validated with the TumorTACS In Situ Apoptosis Detection Kit (R&D Systems, Minneapolis, MN). Necrotic index was determined by quantifying the percent of necrosis over the entire area of tumor, using the software Image-Pro 6.0 (Media Cybernetics, Silver Spring, Maryland).

**Anti-von Willebrand Factor (vWF) Immunohistochemistry**

After sections were deparaffinized, antigen retrieval was performed with Proteinase K treatment for 5 minutes followed by blocking of the endogenous peroxidase activity with a 5 minute treatment with 3% H$_2$O$_2$ (Pharmacy grade). Primary antibody (rabbit anti-human vWF; DAKO, Mississauga, ON) was added at a 1:500 dilution (diluted in 0.1M phosphate buffer supplemented with 5% goat serum as a block) followed with a secondary antibody (Envision +, Goat Anti-Rabbit IgG conjugated to peroxidase, DAKO, Mississauga, ON) and stained with
liquid DAB Substrate-Chromogen System (DAKO, Mississauga, ON) followed by counterstaining with hematoxylin.

The degree of vascularization was assessed by comparing the area of vascularization to the area of the tumor. Areas were digitally quantified by image capture of the entire tumor section with Image-Pro 6.0 software (Media Cybernetics, Silver Spring, Maryland). Viable and nuclear condensed (apoptotic) endothelial cells were counted per sectional area of tumor. Values were reported as number of cells per sectional area of tumor.

Statistical Analysis

Statistically differences between groups were determined by both student’s $T$-test as well as ANOVA assuming single variance.
RESULTS

Efficacy of Co-encapsulation Therapy

Tumor volumes and survival from the treated mice were monitored to assess the efficacy of each treatment. Although each treated group (either singly with IL-2 or angiostatin, or co-treated) was improved significantly from the sham control group, the efficacy of the co-encapsulation therapy was only of intermediate benefit between those of the IL-2 and angiostatic therapies (Fig 1). While the efficacy was enhanced by this double-prong treatment compared to the IL-2 treatment, the lack of synergy over the angiostatin-treatment alone prompted us to ascertain the underlying reason for its failure.

Cell-Encapsulation, Viability and Transgene Expression

We first examined the functional state of the implanted microcapsules. On Day 21 post-tumor cell injection, the capsules from each group were retrieved and assessed for viable cell number and transgene expression (Table 1). Each capsule type had shown an overall increase in cell density per capsule between preimplantation and retrieval, thus confirming that cell proliferation continued after implantation. The capsules from the co-encapsulation protocol had started with approximately double the cell density as the other single-treatment groups (Table 1). However, after the two-week implantation, the cell density within this group of capsules was similar to those of the single-treatment groups.

The transgene expression for each of these respective cell types differed from each other over time as consistent with past studies on the single treatment protocols. The expression of angiostatin from C2C12AST cells increased about twofold; this may represent the improved growth conditions for expression of the angiostatin construct within the peritoneal cavity compared to culture media. However, this was not reflected in the expression of sFvIL-2 from
the C2C12sFvIL-2 cells. Although they retained good viability, the C2C12sFvIL-2 cells have lost their transgene expression (Table 1A). In the capsules containing both cell types, the cell density per capsule remained similar to those of the other groups. However, angiostatin expression has decreased to only about 20% of the preimplantation level, and the sFvIL-2 expression was totally extinguished (Table 1A).

For identification of the genetic origin of the encapsulated viable cells retrieved on day 21, the retrieved microcapsules were cultured in the presence or absence of G418, the selection marker for the original transfected cells (Table 1B). Those cells from the sFvIL-2 and COENCAP groups showed no viability after the 5-day incubation with G418. Indeed, these cells also showed no detectable sFvIL-2 expression. In addition, the cells retrieved from the COENCAP group did not even express angiostatin! In contrast, in the microcapsules containing only C2C12-AST cells, the cells remained viable with continued cell growth and similar levels of angiostatin expression in the presence or absence of G418. Taken together, it is clear that the immunotherapy with the IL-2 fusion protein-secreting cells eventually led to the silencing of transgene expression from all encapsulated cells, including the by-stander angiostatin-secreting cells.

The loss of capsule function in the presence of the IL-2 fusion protein-secreting cells was further corroborated with histopathology and immuno-histochemical staining. Capsules bearing C2C12sFvIL-2 cells (either alone or co-encapsulated with angiostatin-secreting cells) became embedded in adhesions of fibrotic and necrotic mesenteric tissue (Fig 2a: iii & iv). When the embedded capsules were stained histochemically for expression of angiostatin (anti-HA) or IL-2 (anti-hIL-2), no activity could be detected (Fig 2a: vii, viii, xi, xii). In contrast, encapsulated C2C12AST cells did not generate such a response. Hence, usually no embedded capsules could be
found in the mesenteric tissue in the angiostatin-treated group (Fig 2a:ii) or the mock controls (Fig 2a:i).

In contrast to the above-embedded microcapsules, free-floating microcapsules were found in all four groups, but much more in the mock and angiostatin-treated groups. Those from the single angiostatin treatment group contained cells that maintained their viability and transgene expression, as shown from the immunohistochemical staining and bioassay (Fig 2b: ii, v; Table 1). However, those that contained C2C12sFvIL-2 cells indicated a loss of viability and expression (Fig 2b:vii, ix; Table 1). None of cells in these two groups (single IL-2 or co-encapsulated) showed positive immunohistochemical staining for IL-2 (Fig 2b: vii, ix), and only a few of the co-encapsulated cells stained positive for angiostatin (Fig 2b: vi). These observations clearly indicated that the expression of biologically active IL-2 was not compatible with sustained viability and IL-2 transgene expression, and these effects extended even to the co-encapsulated angiostatin-secreting cells.

Systemic Delivery of Therapeutic Products

To test if the distribution of either transgene product was affected by the co-encapsulation, we assayed for sFvIL-2 (Fig. 3) and angiostatin (Fig. 4) in the serum, tumors and peritoneal organs. The immunoconjugate sFvIL-2 was detectable in low quantity (~0.60ng/ml) in serum of the sFvIL-2-treated group while a significantly higher level was observed in the co-encapsulation group (Fig 3a; ~3.4ng/ml sera). In the tissues (Fig 3b), significantly more immunoconjugate was found at the tumors of the co-encapsulation group compared to the sFvIL-2 treatment group. Similarly, a twofold higher level of sFvIL-2 was found in the kidney of the co-encapsulation group compared to the sFvIL-2-treatment group; but the reverse was true for the distribution in the spleen with a higher level of sFvIL-2 in the single treatment group. The
level of the immunoconjugate in the liver was not different between these two groups, both of which were significantly above the background levels of the Mock and Angiostatin-treated controls.

For the delivery of serum angiostatin, although no statistically significant differences were observed among the four treatment groups, wide variations were observed in the Angiostatin-treated and the Co-encapsulation groups (Fig 4a). This may indicate competing forces in trafficking of angiostatin to and from the serum compartment, thus accounting for the wide variations and lack of statistical significance. For the peritoneal organs (Fig 4b: Kidney, Liver and Spleen), no significant differences in angiostatin level were observed among the four groups. In the tumors, however, very high and similar levels of angiostatin were detected in both the Angiostatin-treated- and Co-encapsulated-groups, compared to the Mock and sFvIL-2 treated controls (Fig 4b). These data thus indicated that the immunotherapy encompassed in the co-encapsulation treatment did not affect the angiostatin localization in the tumors or any other compartments examined (Fig 4b).

**Histological Assessment of tumors and intraperitoneal organs**

The anatomical pathology of the tumor and organs indicated a higher level of inflammatory response in those treatments involving the delivery of IL-2. The spleens from COENCAP- and sFvIL2-treated mice showed some follicular activation (hyperplasia) and splenomegaly whereas those from the Mock and Angiostatin-treated groups were unremarkable (Fig 5a). Capsules containing C2C12sFvIL-2 cells only (Fig 5b, sFvIL-2) adhered to the mesenteric tissues. The surrounding inflammation was profuse, characterized by neutrophil infiltration immediately around the capsules, and drastic necrotic damage of the surrounding mesenteric tissue that spanned a long distance from the capsules (hundreds of microns).
However, when co-encapsulated with C2C12AST cells (Fig. 5b, COENCAP), the cytokine-induced necrosis did not reach as far (only in the order of 50-100 microns). Indeed, the necrotic exudates around the co-encapsulated cells occurred only as far as the neutrophils that surrounded the capsule material, while the remaining mesenteric adipose tissue remained healthy. In the Angiostatin- or Mock-treated groups, no capsules aggregated onto the mesenteric tissue although some mesothelial cells became active in the fringes of the mesentery, indicating a mild reaction caused by contact with the capsules (Fig. 5b).

The mesenteric lymph nodes (Fig 5c) of each group were characterized by sinusoidal dilation and follicular hyperplasia, indicating an activation of cellular responses resulting from the capsules per se. This activation appeared similar in all groups, thus indicating that the implanted capsules, regardless of the recombinant product delivered, were the cause of the inflammatory response in the lymph nodes.

Profuse tumor necrosis was observed in the tumors from the various groups but to different extents (Fig 5d). There was prominent formations of lobules separated by laminar septa in all four groups, but with necrosis predominant in the three treated groups only. Quantitative assessment of tumor mitosis, apoptosis and necrosis were performed on H&E stained sections (Fig 6). No significant difference was observed in the mitotic indices among all groups (Fig 6a). Apoptotic indices for each of the treatment groups were greater than that of the Mock control (Fig 6b), while the Co-encapsulation protocol did not showed any increase over the Angiostatin treatment (P > 0.05). However, both these treatments were slightly but significantly superior to that of the IL-2-treated group. This was in contrast to the necrotic indices (Fig 6c) in which the Co-encapsulation and the IL-2-treatment groups have similar scores, both being superior to those
of the Mock or the Angiostatin-treated groups. These last two groups showed no difference from each other.

*Prevalence and Viability of Tumor Endothelial Cells (EC) in Response to Treatment*

EC as identified by anti-vWF staining were assessed in the tumors from the four groups (Fig 7). Interestingly, the number of vWF-positive cells per tumor sectional area was the highest in the mice receiving the Co-encapsulation treatment (Fig 7a; P < 0.05), whereas those values from both the single treatment groups were similar to that of the Mock control. However, when apoptosis was assessed in these EC cells, as indicated by nuclear condensation, dissociation of vessel structure and formation of apoptotic bodies in the cells positively stained for vWF, the groups receiving angiostatin alone or in combination with the immunotherapy induced the highest and similar levels of tumor-derived endothelial cell apoptosis (>25%), whereas the IL-2-treated group only had a minor although statistically significant increase in endothelial cell apoptosis compared to the Mock control (~14% cf. ~10%, respectively, P < 0.05).

*Vascular Mimicry*

In the course of the above assessment of the EC status in tumors, it was noted that vascular channels containing erythrocytes can be categorized into three classes, depending on the continuity of the lining von-Willebrand-positive EC cells (Fig 8): an “ALL EC” type in which the vascular channel was lined by a continuous layer of EC; a “Partial EC” type in which the lining of EC was discontinuous; and a “NO EC” type in which a EC lining was absent, leaving a direct interface between the erythrocytes and tumor cells. The relative contributions of these three vascular types were found to differ. The most outstanding difference was found in the Angiostatin-treated group with the lowest “All
EC” type and the highest “No EC” type among the four groups. However, this trend was reversed in the COENCAP group in which the “All EC” type was comparable to that of the Mock control, while the “No EC” type was much reduced, even though it was still significantly above the level of the Mock control. The sFvIL-2 single treatment group showed no difference from the Mock control in all three categories as expected.
DISCUSSION

Although our early application of the microencapsulation technology for cancer treatment was successful in suppressing tumor growth when IL-2^{10} or angiostatin^{30} was used alone, co-encapsulating the two producer cell types in the same microcapsules in the current protocol did not lead to a synergistic efficacy (Fig 1). In fact, the Co-encapsulation protocol, while superior to the IL-2 single treatment, was less effective than the single Angiostatin treatment. Further analyses showed that the inflammatory response driven by the expression of sFvIL-2 led to the loss of viability and transgene expression, not only in the IL-2 expressing cell line, but also the angiostatin-secreting cells that were co-encapsulated (Table 1b). It was clear that by Day-21 post injection of tumors cells (14 days after the implantation of encapsulated cells), very few cells of either type in these microcapsules delivered any therapeutic benefit. The cells within the co-encapsulated capsules were a mixture of angiostatin-secreting and non-producer cells. It is likely the C_{2}C_{12}AST cells, being in close proximity to the C_{2}C_{12}sFvIL-2 cells, were susceptible to the same silencing mechanism, rendering some of these once angiostatin-secreting cells non-expressing as well. In contrast, the angiostatin-treated group showed sustained delivery of angiostatin (Table 1B) and continued to provide therapeutic benefit, accounting for the more sustained suppression of tumor growth and prolonged survival (Fig. 1).

The concurrent delivery of IL-2 and angiostatin also has influenced their distribution systemically. The distribution of the IL-2 fusion protein was particularly interesting. When delivered concurrently with angiostatin, IL-2 accumulated less in the spleen while more was found in the kidneys, the tumor, and systemically (Fig 3a, b). This may be related to angiostatin’s ability to alter neutrophil trafficking^{34} or adhesion molecule expression on endothelial cells. Angiostatin, when bound to integrins^{35}, may alter trafficking of lymphocytes
while the expression of hypoxia-induced proangiogenic factors in tumors can alter the expression of cell adhesion molecules involved in systemic cell trafficking\textsuperscript{36,37}. Hence, this alteration by angiostatin in the trafficking of cells that have receptors for IL-2 may affect how IL-2 is distributed throughout the host. The significantly higher level of sFvIL-2 in the circulation and in the tumor may account for the improved efficacy of the co-encapsulation protocol compared to the single IL-2 treatment (Fig 3b).

As for the angiostatin, it was localized mainly to the tumor, and its distribution was not affected by the co-delivery of IL-2 (Fig 4b). However, the effect of angiostatin \textit{in vivo} on endothelial cells was attenuated, with both negative and positive consequences. There was an increase in density of vWF-positive cells in the tumors from the Co-encapsulated group, likely due to the increased proliferation or decreased apoptosis of tumor endothelial cells compared to the Angiostatin-treatment alone (Fig 7a). This may have accounted for some of the loss in efficacy in the Co-encapsulation group (Fig 1). However, we had previously shown that tumors from mice treated with angiostatin have increased level of vascular channels without endothelial cell lining, a phenomenon we described as vascular mimicry\textsuperscript{30}. In this study, the same phenomenon was observed in the Angiostatin-treated group but it was much reduced in the Co-encapsulation group (Fig 8: No EC). Hence, the co-encapsulation strategy with concurrent immune therapy did show some success in subduing the vascular mimicry induced by angiostatin. Since this may be an escape mechanism by tumor cells from anti-angiogenic treatment, the Co-encapsulation thus provides an improvement over the single Angiostatin-treatment group.

In conclusion, cancer therapy with cytokines and angiostatin via encapsulated cells has been successful in this model system, either alone or together. While there was no synergy in
efficacy in the Co-encapsulation protocol, there was some incremental advantage and significant reduction in undesirable side effects. Future directions in using encapsulated cells for cancer treatment will likely build on similar multi-prong approaches involving different anti-cancer mechanisms\textsuperscript{39}, including those in current clinical practices. With appropriate optimization, this should lead to long term efficacy and amelioration of adverse side effects.
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References


Table 1: Cell Viability and Transgene Expression of Encapsulated Cells. Viability and transgene expression of the encapsulated cells were assessed in each group from microcapsules that were free within the peritoneal cavity. (A) The encapsulated cells were assayed for viability as well as angiostatin or sFvIL-2 expression prior to implantation on day-0 (Preimplant) and upon retrieval from mice on Day-21 post tumor-cell injection (Retrieved). Transgene expression was determined in the media collected after incubating the microencapsulated cells for 24 hours. B) Retrieved capsules were incubated for 5 days with and without G418 selection before viability and expression analyses. N/A: No Activity detected. Data represent averages from triplicate assays from N = 3 mice for the retrieved samples±SEM. *: P < 0.05, **: P < 0.01.
Legends to Figures

Figure 1 Efficacy of Co-encapsulation Therapy.

Four groups of mice (N = 7-23 mice per group from 3 independent experiments) were injected with B16-F10/neu tumor cells on Day-0. On day-7, the Mock-treated control group received encapsulated but untransfected C2C12 cells. Single treatment groups received encapsulated cells secreting either angiostatin (C2C12-AST) or sFvIL-2 (C2C12-sFvIL-2). The remaining COENCAP group received both cell types co-encapsulated together (See M&M). (a) Tumor volumes of subcutaneous B16-F10/neu tumors were measured up to Day-21. (b) Kaplan-Meier plot of animal survival (end point defined by tumor volume reaching 1cm$^3$). Significant differences were obtained at Day 21 between the experimental group and the three control groups (*: P < 0.05, **: P < 0.01, ***: P < 0.001 respectively) as determined by student’s T-test and confirmed by ANOVA.

Figure 2: Immunohistochemical assessment of encapsulated cells.

Post-mortem examination of the peritoneal cavities of mice at Day 21 showed microcapsules either within inflammatory adhesions localized to lymphoid tissue of the mesentery (a) or unbound and freely floating throughout the peritoneal space (b). Immunohistochemical staining against the HA tag of the recombinant angiostatin (Anti-HA; Texas Red) or against the human IL-2 component of sFvIL-2 (Anti-rhIL-2; FITC Green) were performed. (a) Microcapsules embedded within adhesions were examined from parafin-embedded mesenteric tissue sections and yielded no positive stains in any of the treatment groups (N=5 mice per group, triplicate sampling per mouse). Parental cell lines were used as positive controls (data not shown). (b)
Cells released with citrate from free-floating capsules were plated onto slides and showed only recombinant angiostatin but no sFvIL-2 expression. Dashed lines indicate microcapsule outer rim (iii, iv).

**Figure 3 Delivery of sFvIL-2.**
sFvIL-2 in serum (a) as well as tumor and various intraperitoneal organs (b) was determined on Day-21 post tumor cell-injection. (N = 5 mice per group; data represent triplicate from each of 2 independent experiments).

*: P < 0.05

**: P < 0.01

**Figure 4 Delivery of Angiostatin.**
Angiostatin in serum (a) as well as in peritoneal tissues and tumor (b) was determined on Day-21 post tumor-injection. N = 5 mice per group; data represent triplicate data over 2 independent experiments.

*: P < 0.05

**Figure 5 sFvIL-2 and Angiostatin Delivery to Organs and Tumors.**
Spleen (a), mesentery (including capsules whenever possible) (b), mesenteric lymph nodes (c) and tumors (d) from each of the four groups of mice obtained post-mortem on Day-21 were processed for H&E staining. Representative fields of the tissue sections are presented. Bars for Spleen and Lymph node pictures represent 1mm; Bars for Mesentery and Tumors represent 500µm.
Figure 6 Histological Analysis of Tumor Tissues.

H&E stained tumor sections from the four groups of mice were analyzed morphometrically to yield mitotic (a), apoptotic (b), and necrotic (c) indices. (N = 5 mice / group; *: P < 0.05, **: P < 0.01).

Figure 7 Histological Examination of Tumor Endothelial Cells.

Tumor sections were stained for vWF-positive endothelial cells (EC) and analysed morphometrically. Number of vWF-positive cells per tumor sectional area (a), and assessment of apoptotic vWF-positive cells (b) were performed. N = 5 mice / group with error bars representing SEM. (*: P < 0.05, **: P < 0.01 compared to the Mock controls unless otherwise indicated).

Figure 8 Vascular Mimicry.

The tumor sections from the four groups of mice were stained for vWF, and tumor vasculature was sorted according to the endothelial cell content surrounding the observed vessel. Vasculature observed to consist of a continuous layer of endothelial cells (vWF positive) were categorized in 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 were also analyzed (“Partial EC”). Entire tumor sections were examined under several medium fields of view and the surface area of the vasculature relative to the tumor sections was assessed with morphometrical analysis using ImagePro 6.0 software (see M&M). N = 5 mice per group (5 non-serial sections per mouse).
Error bars represent the SEM. (*: $P < 0.05$, **: $P < 0.01$ compared to the Mock controls unless otherwise indicated).
Figure 1 Efficacy of Anti-Angiogenic Therapy with Immunotherapy.

Graph a: Tumor Volume (mm³) vs. Days Post Tumor-Cell Injection

Graph b: Survival vs. Days Post Tumor Cell Injection

Legend:
- MOCK
- Angiostatin
- sFvIL-2
- COENCAP

Statistical significance:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
Figure 2: Immunohistochemical assessment of encapsulated cells in mice.

**Phase Contrast**

- MOCK
- Angiostatin
- sFvIL-2
- COENCAP

**anti-HA**

- i
- ii
- iii
- iv

**anti-hIL-2**

- v
- vi
- vii
- viii

The figure compares the immunohistochemical staining for HA and hIL-2 in different conditions. The images show the distribution of these proteins in encapsulated cells from MOCK, Angiostatin, sFvIL-2, and COENCAP conditions.
Figure 3 Systemic Delivery of sFvIL-2.

(a) and (b) show the systemic delivery of sFvIL-2 in different tissues.

(a) Graph comparing MOCK, Angiostatin, sFvIL-2, and COENCAP:
- MOCK (M)
- Angiostatin (A)
- sFvIL-2 (S)
- COENCAP (C)

(b) Graph showing sFvIL-2 levels in Tumor, Kidney, Liver, and Spleen:
- Tumor
- Kidney
- Liver
- Spleen
Figure 4 Systemic Delivery of Angiostatin.

(a) Angiostatin (µg/ml sera)

(b) Angiostatin (ng µg protein)

- MOCK (M)
- sFvIL-2 (S)
- Angiostatin (A)
- COENCAP (C)
Figure 5 Tissue Histology of Treated Animals.

(a) Spleen  (b) Mesentery  (c) Lymph Node  (d) Tumor

Mock

Angiostatin

s-Fv/IL-2

COENCAP

1mm

500μm

1mm

500μm
Figure 6 Histological Analysis of Tumor Tissues.

(a) Mitotic Index (%)

(b) Apoptotic Index (%)

(c) Necrotic Index (%)

- Mock
- sFvIL-2
- Angiostatin
- COENCAP

Significance levels: **p < 0.01, ***p < 0.001, (-) non-significant
Figure 7 Histological Examination of Tumor Endothelial Cells.

(a) Total EC/Tumor Area (Cell #/mm²)

- Mock
- Angiostatin
- sFvIL-2
- COENCAP

(b) EC Apoptotic Index (%)

- Mock
- Angiostatin
- sFvIL-2
- COENCAP

* and ** indicate statistical significance.
Figure 8 Vascular Mimicry.

- **Mock**
- **Angiostatin**
- **sFvIL-2**
- **COENCAP**

**Vascular Area / Tumor Sectional Area**

- **ALL EC**
- **Partial EC**
- **NO EC**

* Statistical significance indicated by asterisks.