[CANCER RESEARCH 59, 4668–4674, September 15, 1999]

Sensitization of Tumor Necrosis Factor α-resistant Human Melanoma by Tumor-specific *in Vivo* Transfer of the Gene Encoding Endothelial Monocyte-activating Polypeptide II Using Recombinant Vaccinia Virus[1]

Michael F. X. Gnant, Adam C. Berger, James Huang, Markus Puhlmann, Peter C. Wu, Maria J. Merino, David L. Bartlett, H. Richard Alexander, Jr., and Steven K. Libutti[2]

Surgery Branch [M. F. X. G., A. C. B., J. H., M. P., P. C. W., D. L. B., H. R. A., S. K. L.] and Surgical Pathology Section [M. J. M.], National Cancer Institute, NIH, Bethesda, Maryland 20892; Department of Surgery, University of Vienna, A-1090 Vienna, Austria [M. F. X. G.]; and Department of Surgery, Technical University Munich, D-81675 Munich, Germany [M. P.]

ABSTRACT

Tumor necrosis factor α (TNF-α) is a proinflammatory cytokine with potent experimental antitumor activity. Its clinical use in cancer treatment is severely limited by its considerable toxicity after systemic administration, and it is currently confined to isolated limb and organ perfusion settings.

In this report, we introduce a novel concept of TNF-α-based gene therapy using the TNF-sensitizing properties of endothelial cell monocyte-activating polypeptide II (EMAP-II). We hypothesized that transfer of the *EMAP-II* gene into established TNF-resistant human melanomas would render these tumors sensitive to subsequent systemic TNF-α treatment.

To achieve tumor selective gene delivery, we constructed a recombinant vaccinia virus encoding the human *EMAP-II* gene (vvEMAP). *In vitro* transfection of human melanoma cells led to the production of EMAP-II by these cells. Supernatants of vvEMAP-transfected tumor cells mediated the induction of tissue factor in endothelial cells.

We characterized the pattern of gene expression after systemic administration of a recombinant vaccinia virus encoding a reporter gene in a murine *in vivo* model of s.c. human melanoma. Gene expression in tumor tissue was increased 100-fold as compared with normal tissue, providing evidence for tumor-selective gene delivery.

Finally, human melanomas in nude mice were sensitized *in vivo* by transferring the *EMAP-II* gene using vvEMAP. Subsequent systemic administration of TNF-α led to tumor regression and growth inhibition of these previously TNF-resistant tumors ($P < 0.05$).

This approach using gene therapy to sensitize primarily unresponsive tumors toward TNF-α may enhance the usefulness of TNF-α in clinical treatment strategies by increasing the window for the therapeutic application of the cytokine, thus reducing the dose necessary for antitumor responses and subsequently reduce toxicity.

INTRODUCTION

TNF-α[3] was initially described and named for its ability to induce necrosis in certain types of malignant tumors (1). The mechanism by which the cytokine exerts this effect is still not completely understood; however, experimental and clinical evidence shows that the effects of TNF-α on tumor-related vasculature are the most important component of its antitumor activity (2–4).

Clinical responses induced by systemic TNF-α are unusual in melanoma patients (5, 6), and its therapeutic use never came close to its initial expectations, based on preclinical data. The reasons for this are mainly related to the toxicity of the cytokine (7–9). TNF-α has numerous proinflammatory activities inducing capillary leak syndrome, shock, and coagulation disorders following systemic administration (10, 11), at doses far below those required to mediate antitumor effects (12).

There is, however, still clinical use of TNF-α in treatment settings in which systemic toxicity can be avoided by anatomical isolation and perfusion of a cancer-bearing organ or region of the body (13, 14). Several groups, including ours, have described the usefulness of this approach in isolated limb and hepatic perfusion for extremity melanoma, sarcoma, or unresectable liver tumors (15–18). Dramatic responses can be observed quite frequently (19), and the procedures have been well defined and can eliminate systemic exposure to TNF-α (14). Currently, clinical trials are underway to define the exact contribution of TNF-α to these treatments as well as the optimal schedule and dosage (20, 21). Most, if not all, of the observed antitumor effects of the cytokine are achieved indirectly via its effect on the vasculature, with increased capillary permeability, erythrostasis, and endothelial cell necrosis (22, 23).

There have been several approaches to develop TNF-α-related gene therapies, mostly by introducing the gene for the cytokine into effector cells or tumor cells using an adoptive immunotherapy or tumor vaccine approach (24–29). Most reported *in vivo* results were disappointing, either because of low effectiveness or subsequent toxicity (30–32).

The tumor-derived cytokine EMAP-II is an important factor in the activation of tumor neovasculature (33). EMAP-II is translated as a M_r 34,000 precursor protein and cleaved to a M_r 22,000 mature protein (34). High EMAP-II expression leads to induction of tissue factor in endothelial cells and correlates directly with the TNF-α sensitivity of tumors (35). Unfortunately, whereas both the *EMAP-II* gene and mRNA is present in all eukaryotic cells (36), protein expression is down-regulated in mature tissues as well as in most tumors. Actual protein expression levels differ considerably among tumors, even of the same histological type, and their respective sensitivities toward *in vivo* treatment with TNF-α depend on this expression. Both intratumoral injection of recombinant EMAP-II protein into TNF-resistant tumors (37) and stable *in vitro* transduction of low EMAP-II-expressing melanomas (35) rendered those tumors TNF-α sensitive *in vivo*, adding further evidence to the direct relationship between EMAP-II expression and TNF-α sensitivity.

Although these observations helped elucidate the mechanisms of TNF-α activity and its interaction with EMAP-II, their potential clinical applicability is limited, mainly because most tumors are low EMAP-II expressers. We hypothesized that the *in vivo* introduction of the *EMAP-II* gene into TNF-α-resistant tumors might lead to increased EMAP-II production by these tumors, thus providing a window of opportunity for systemic TNF-α treatment. By gene transfer leading to overexpression of EMAP-II specifically within tumors, we would be able to artificially increase the therapeutic window for TNF-α between these tumors and normal tissue, thus facilitating the

Received 2/10/99; accepted 7/20/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] M. F. X. G. is a recipient of a research grant from the Max Kade Foundation (New York, NY).

[2] To whom requests for reprints should be addressed, at Surgical Metabolism Section, Surgery Branch, National Cancer Institute, NIH, Building 10, Room 2B07, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-5049; Fax: (301) 402-1788; E-mail: Steven_Libutti@nih.gov.

[3] The abbreviations used are: TNF-α, tumor necrosis factor α; EMAP-II, endothelial monocyte-activating polypeptide II; TK, thymidine kinase; HUVEC, human umbilical vein endothelial cell; IL, interleukin; rlu, relative light unit(s).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch1899004668p.pdf by guest on 03 March 2024

use of nontoxic treatment dosages. The vector system used here is a recombinant vaccinia virus that is severely attenuated by the disruption of its TK gene (38).

Plasmids and viruses carrying the *EMAP-II* gene or a reporter gene were synthesized, and *in vitro* experiments provided evidence of successful and selective gene transfer and expression in tumor cells. We then characterized the expression of a reporter gene in a murine *in vivo* model of s.c. human melanoma in tumors compared with other tissues over time. Finally, we describe the *in vivo* application of the sensitizing gene therapy and subsequent systemic TNF-α treatment and its effect on *in vivo* tumor growth.

MATERIALS AND METHODS

Cell Lines. Pmel and 1286 are primary human melanoma lines derived from patients treated at the National Cancer Institute (Bethesda, MD). The human embryonic kidney cell line 293, the ovarian cancer line HeLa, and the monkey kidney cell line CV-1 (American Type Culture Collection, Manassas, VA) were used for generation, amplification, and titration of recombinant vaccinia viruses. All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin, 1% gentamicin, and 0.2% fungizone (Biofluids, Rockville, MD) at 37°C in a 5% CO_2 incubator.

HUVECs (Clonetics, Walkersville, MD) were maintained at 37°C in a 5% CO_2 incubator in EGM-2 medium (basal endothelial cell media EBM enriched with hydrocortisone, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor-1, ascorbic acid, epidermal growth factor, GA-1000, and heparin) available from Clonetics. They were passaged no more than six generations and cryopreserved at regular intervals.

Construction and Cloning of Plasmids and Viruses. The *EMAP-II* gene was amplified from human monocyte cDNA via PCR using *Pfu* polymerase (Stratagene, La Jolla, CA) and using primers derived from the published GenBank sequence (accession no. U10117). After ligation into the pCRII cloning vector (Invitrogen, Carlsbad, CA), the correct gene sequence was confirmed by automated sequencing (ABI Prism, Perkin-Elmer Applied Biosystems, Norwalk, CT). The human *EMAP-II* gene was then cloned by directional cloning into the vaccinia virus shuttle vector vCB023-II, resulting in pMPhEII. The human *EMAP-II* is therein driven by the optimized synthetic early-late promoter. The construction of vCB023-II is described in detail elsewhere (39). Briefly, the *Xba*I site of pSC65 (kindly provided by B. Moss, NIH, Bethesda, MD) within the flanking region of the TK gene was abolished with Klenow and dNTP filling. The plasmid was then amplified with primers to create a *BSSH II* site between the TK flanking regions, allowing for the insertion of the multiple cloning site of pBluescript KS II (+) (Stratagene, La Jolla, CA), which was used to insert *gpt* as well a promoter cassette consisting of a vaccinia virus p7.5 early/late and a synthetic early/late promoter (40, 41) in the sense and antisense directions, respectively, immediately upstream.

A plasmid containing the firefly luciferase gene was constructed in a similar way using the luciferase gene from pGEM-luc (Promega, Madison, WI), as described previously (39).

Recombination of vaccinia viruses (strain Western Reserve) was performed using CaCl precipitation (42). Recombinant vaccinia viruses were subsequently isolated by mycophenolic acid selection and designated vvLuc for the reporter gene-containing virus and vvEMAP for the virus containing the *EMAP-II* gene. Recombinant viruses were amplified in HeLa S3 cells using spinner flasks as described (42) and titered in standard plaque forming assays using CV-1 cells.

Extraction of RNA and PCR Amplification. Total RNA was extracted from cell cultures infected with the viral constructs using RNEasy (Qiagen, Chatsworth, CA). cDNA was transcribed by reverse transcriptase reaction, and PCR was performed. For vvEMAP, the following primers were used: 5′-AACTGAAACAAGAGCTAATT-3′ and 5′-CAGGCTCTCCTGGGAAAG-CA-3′. Amplification of cDNA was performed using a GeneAmp thermocycler (Perkin-Elmer) using 25 cycles of a 94°C denaturing step (60 s), a 52°C annealing step (30 s), and a 70°C extension step (90 s).

Preparation of Tumor-conditioned Medium for *in Vitro* Experiments. To produce tumor-conditioned medium, we plated $\sim 5 \times 10^6$ tumor cells per 75-cm^2 tissue culture flask (Corning Costar, Cambridge, MA) in 15 ml of serum-free medium 199 (NIH Media Unit, Bethesda, MD). After 24 h, Pmel tumor cells were transfected with vvEMAP or vvLuc (control virus) at a multiplicity of infection (MOI) of 0.1 for 20 h. Conditioned medium was then collected and vacuum-filtered across a 0.45-μm membrane (Corning) to remove any cellular debris and used immediately.

Tissue Factor Induction Assay. HUVECs were plated at 5×10^5 cells per well of a six-well plate (Corning). After 48 h, the cells were washed twice with sterile PBS and treated, in triplicate, with 2 ml of tumor-conditioned medium, medium conditioned by tumors infected with the viral constructs, EGM-2 medium alone (negative control), or EGM-2 medium plus 150 ng/ml IL-1β (R&D Systems, Minneapolis, MN) as a positive control. In addition, one group of HUVECs was treated with vvEMAP-conditioned medium plus 200 μg/ml polyclonal EMAP-II rabbit IgG as an EMAP-II-blocking antibody. The treated endothelial cells were cultured at 37°C for 18 h, washed twice with sterile PBS, harvested in 500 μl of PBS per well using mechanical scrapers, lysed using three rapid freeze-thaw cycles, and stored at −70°C.

Cell suspensions were thawed at 37°C and centrifuged at 2000 × *g* for 5 min. The cell pellet was then resuspended in 200 μl of 50 nM Tris, 100 nM NaCl, and 0.1% BSA. A one-stage coagulation assay was performed by adding 100 μl of Factor VIII-deficient human plasma (George-King Biomedical, Overland Park, KS) to 100 μl of endothelial cell suspension and incubating for 2 min at 37°C. The reaction was catalyzed by the addition of 100 μl of 30 nM $CaCl_2$; clotting time was measured using an Amelung micro-coagulation analyzer (Sigma, St. Louis, MO). Standard curves were generated using dilutions of recombinant human tissue factor (American Diagnostica, Greenwich, CT). The assay sensitivity for TF was <10 pg/ml.

Animal Care and Tumor Model. Female athymic NCR-nude mice, 8–12 weeks old, were obtained from the NIH small animal facility and housed in a barrier care room. Human tumors were inoculated in the right flank region by s.c. injection of 10^6 tumor cells suspended in 200 μl of sterile HBSS. Animals had unlimited access to food and water and were housed at a maximum of five mice per cage. All animal protocols were approved by the Animal Care and Use Committee and conducted in strict compliance to the guidelines established by the NIH Animal Research Advisory Committee.

For the reporter gene experiments, animals were injected i.v. with vvLuc when tumors reached $\sim$300 mm^3 in size, typically 3–4 weeks after tumor inoculation. Cohorts of animals were sacrificed at various time points and tumors and normal tissues (liver, kidneys, spleen, uterus, ovary, bowel, heart, lung, muscle, skin, and brain) were harvested, immediately frozen, and stored at −70°C.

For the *in vivo* treatment experiments tumor sizes were measured 21–28 days after the injection of tumor cells. Animals were ear-tagged and randomized at that time point, and identical average tumor sizes (close to 300 mm^3) were selected for the actual experimental cohorts. The viral constructs were injected via tail vein injection in 200 μl of HBSS. Daily tumor measurements using a caliper were performed by blinded investigators, and tumor volumes were calculated according to the formula: volume = $width^2 \times length \times 0.52$ (43). On days 4 and 6 after virus injection, treatment groups received the maximum tolerable dose for systemic administration, 7 μg of TNF-α (Knoll Pharmaceutical, Whippany, NJ), via tail vein. Activity of a given TNF-α lot was ensured using concomitant *in vivo* treatment of TNF-α-sensitive 1286 tumors (data not shown). Control groups received tail vein injections of the vehicle alone (200 μl of 0.9% NaCl solution-0.5% BSA). Animals were continuously measured in a blinded way on a daily basis until tumor sizes reached 1000 mm^3. At that point, animals had to be sacrificed according to protocol and guidelines to avoid prolonged pain and suffering.

Firefly Luciferase Assay. Luciferase activity was assayed using a commercially available assay system (Promega). Frozen tissue samples were briefly thawed, homogenized, and lysed in 750 μl of 1× reporter gene lysis buffer. Samples were centrifuged for 5 min at 13,000 rpm to pellet the cellular debris. Ten μl of supernatant were added to 100 μl of luciferase assay reagent in an 8 mm × 50 mm disposable cuvette and immediately placed into a luminometer (model TD-20/20; Turner, Sunnyvale, CA). After a 2-s delay, light emission was measured for 10 s. Each sample was measured in duplicate, and mean values were recorded. The concentration of total protein in each sample was then determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using a BSA standard. All measurements were performed in triplicate. The final firefly luciferase activity is expressed in rlu (= light units per mg protein).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch18990004668p.pdf by guest on 03 March 2024

Histology and Immunohistochemistry. Tumor and tissue specimens were harvested from animals sacrificed at time points given and immediately fixed with 10% paraformaldehyde in 0.1 M phosphate buffer. Paraffin embedding, sectioning, and H&E staining were performed at Paragon Biotech (Baltimore, MD). Immunohistochemistry for EMAP-II was performed using a polyclonal chicken anti-EMAP-II antibody at a dilution of 1:4000 (Aves Laboratories, Tigard, OR) and horseradish peroxidase detection. Slides were examined using light microscopy and read by an investigator blinded to the treatment groups.

Statistical Analysis. Data were analyzed using ANOVA with repeated measures and Tukey post hoc tests using Systat (Evanston, Illinois) statistical software and are given as means ± SE. When appropriate, Student's paired *t* test (two-sided) was used, and means ± SD are reported.

RESULTS

Transfer of the *EMAP-II* Gene into Human Melanoma Cells *in Vitro* Leads to Functional Protein Production. We developed a functional bioassay of EMAP-II activity based on the induction of tissue factor in human endothelial cells by EMAP-II-transfected tumor cells. HUVECs were treated with medium conditioned by tumor cells infected with vvEMAP and vvLuc. As shown in Fig. 1, a 20-h exposure of HUVECs to medium conditioned by Pmel infected with vvEMAP leads to an 8-fold increase in the production of tissue factor by these cells, as compared with treatment with conditioned medium from control Pmel or from Pmel infected with vvLuc ($P < 0.001$). The addition of anti-EMAP-II antibody abrogated the activating effect of medium conditioned with vvEMAP-infected cells (nonsignificant *P versus* Pmel controls; $P < 0.01$ *versus* Pmel plus vvEMAP).

Pattern of Reporter Gene Expression *in Vivo* after Systemic Vector Delivery. The tumor model used for our *in vivo* studies was generated by s.c. injection of nude athymic mice with TNF-α-resistant Pmel human melanoma cells. Tumors were grown to an approximate size of 300 mm^3, and 10^6 pfu of vvLuc in 200 μl of HBSS were administered systemically via lateral tail vein injection. HBSS injections were used as negative controls.

Groups of mice ($n = 5$) were sacrificed at days 1, 3, 5, 7, 9, 12, 14, 16, 18, and 21 after virus injection. Tumor and tissue samples were harvested and assayed for their luciferase content and normalized to the total protein content of samples. Fig. 2 shows the results expressed in rlu per mg protein. In general, tumor samples had a gene expression that was ~100 times higher than those of other tissues, providing evidence of tumor-selective delivery of the foreign gene. Maximum gene expression in tumors was observed between days 4 and 7, and the difference in luciferase activity between tumors and the next closest gene-expressing organs was maintained for up to 21 days. The observed pattern in gene expression proves the existence of a "therapeutic window," encouraging the usefulness of the approach in the treatment setting.

TNF-α Treatment of Tumors *in Vivo* after Systemic Pretreatment with vvEMAP. The human melanoma model in nude mice was used for *in vivo* treatment experiments. According to the gene expression pattern observed in the reporter gene experiments, treatment with TNF-α was scheduled on days 4 and 6 after systemic delivery of vvEMAP. Control cohorts included mice treated with PBS or vvLuc. TNF-α was administered to animals at a dose of 350 μg/kg body weight via lateral tail vein injection. Negative treatment controls received tail vein injections of the vehicle alone. Treatment and tumor measurement were performed in a blinded fashion.

Fig. 3 shows the tumor volume of animals treated with TNF-α after pretreatment with vvEMAP or vvLuc or PBS. Starting on day 7, a significant difference in tumor volumes was observed (day 7, $P = 0.04$). Whereas control tumors pretreated with vvLuc or PBS continued to grow uninhibitedly despite TNF-α treatment, TNF-α abrogated any further growth in the previously TNF-resistant Pmel tumors, indicating the functionally effective overexpression of the sensitizing *EMAP-II* gene in these tumors. This difference was observed until the end of the experimental period and increased markedly over time (day 14, $P < 0.0001$). The effect was observed irrespective of the number and timing of TNF-α treatments (data not shown). There was no difference between vvLuc- and PBS-pretreated tumors (nonsignificant *P* at all time points). All negative control subgroups treated with vehicle instead of TNF-α, irrespective of vector pretreatment showed continued tumor growth (data not shown).

Histopathology and Immunohistochemistry. Successfully treated tumors showed the typical macroscopic and microscopic effects of TNF-α. As an example, Fig. 4*A* shows hemorrhagic necrosis macroscopically observed in vvEMAP/TNF-α tumors as compared with control vvLuc/TNF-α tumors (Fig. 4*B*). Using light microscopy, we determined the average percentage of tumor necrosis in vvEMAP/TNF-α tumors to be ~75–85% *versus* 20–30% in controls (Fig. 4, *C*

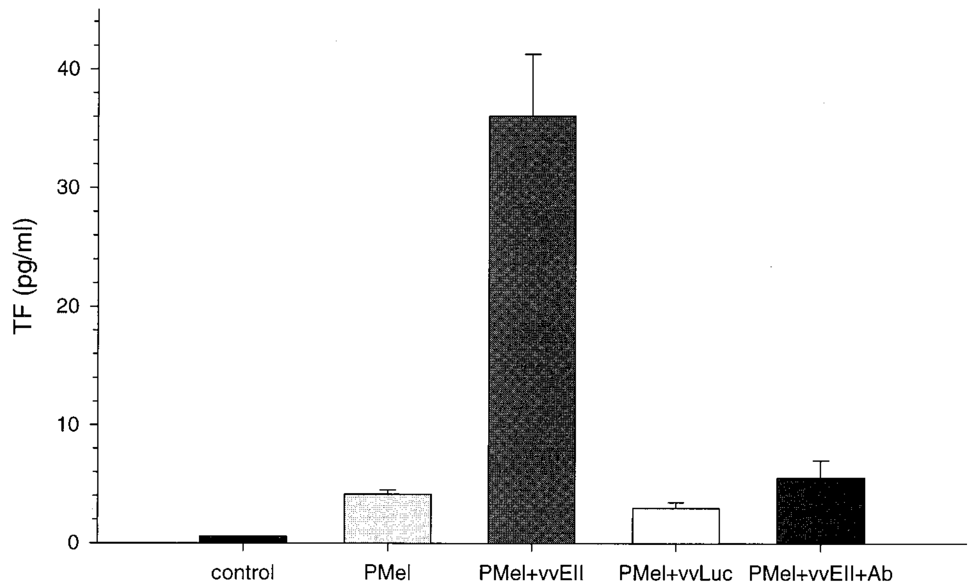

Fig. 1. Tissue factor expression of HUVECs *in vitro* after treatment with conditioned medium from tumor cells infected with vvEMAP. HUVECs were treated with medium conditioned by melanoma cells infected with viral vectors. Transfection of Pmel with vvEMAP yields functional EMAP-II production, indicated by the 8-fold increased induction of tissue factor in HUVECs as compared with untransfected Pmel ($P < 0.001$) or cells transfected with vector control alone (vvLuc). Blocking of EMAP-II with specific anti-EMAP-II antibody abrogates this effect (nonsignificant *P versus* Pmel; $P < 0.01$ *versus* Pmel plus vvEMAP).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch18990004668p.pdf by guest on 03 March 2024

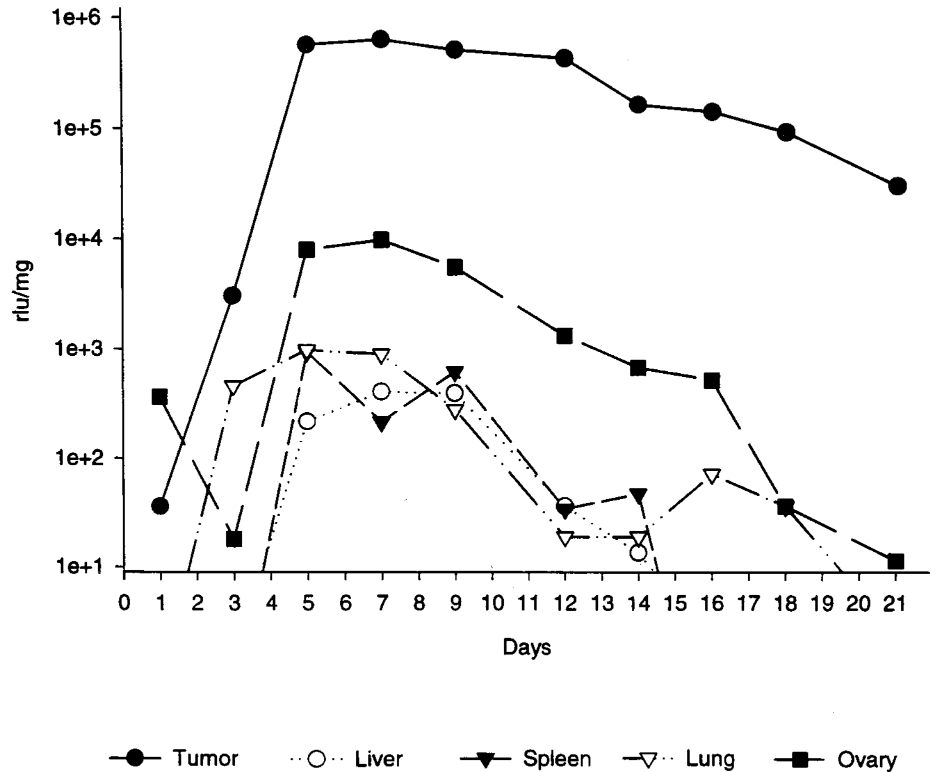

Fig. 2. Pattern of luciferase expression in tumors *versus* normal tissues after systemic delivery of vvLuc. Nude athymic mice bearing human s.c. Pmel melanomas (size, ~300 mm^3) were injected with 10^6 pfu vvLuc via lateral tail vein injection. Groups of five animals were sacrificed at time points given, and tumor and selected tissues were harvested and processed as described. Luciferase content is given in rlu per mg total protein. *Data points*, means of five animals per time point. Gene expression in additional organs was determined (data not shown) and was found to be <10 rlu/mg at all time points.

and *D*, respectively). Even in treatment animals, however, a small rim of viable tumor cells survived and led to eventual regrowth of tumors.

Histopathological analysis of other organs, particularly the ovaries, did not show necrosis or other changes.

Immunostaining for the presence of EMAP-II protein in tumors treated with vvEMAP showed a marked cytoplasmic and membrane-bound staining pattern (Fig. 4*E*), as compared with controls treated with vvLuc (Fig. 4*F*).

DISCUSSION

We have constructed a recombinant vaccinia virus expressing the human *EMAP-II* gene and shown the feasibility of tumor-specific gene delivery in a murine model of s.c. human melanoma using this vector system. Functional protein secretion was achieved *in vitro* and *in vivo* and led to the sensitization of a previously TNF-α-resistant tumor toward TNF-α treatment.

The clinical use of TNF-α has been disappointing, mainly because it is impossible to deliver the necessary antitumor doses of the cytokine to patients without severe toxic side effects. Systemic TNF-α has been very effective in the treatment of established murine tumors (44), but mice tolerate as much as 50-fold higher relative TNF-α dosage than humans (45). The specific targeting of tumors with the cytokine is impossible in most clinical settings, including generalized melanoma, due to the difficulty of tumor-specific delivery of the agent

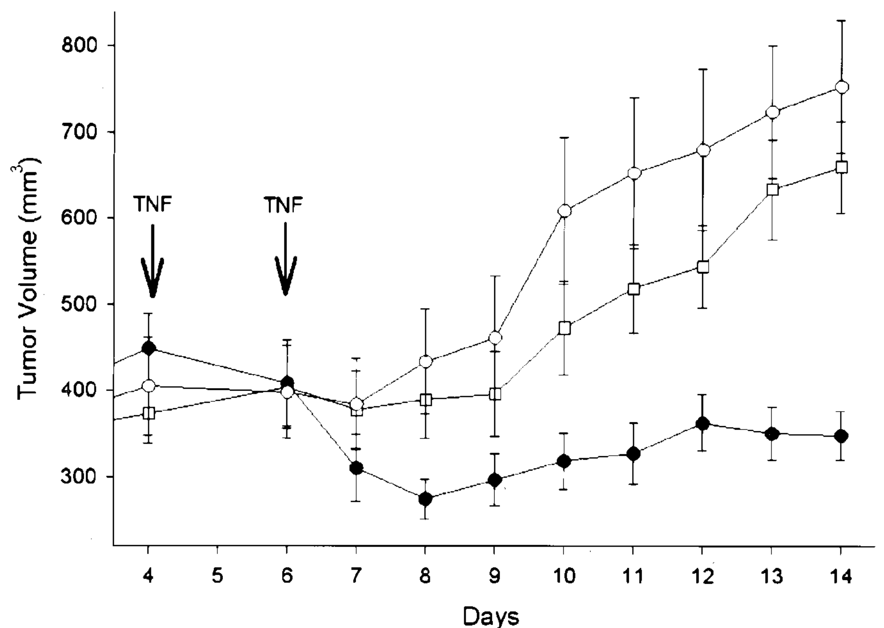

Fig. 3. Tumor volumes after pretreatment with vectors and TNF-α treatment. At day 0, tumor bearing mice were systemically injected either with vvEMAP, vvLuc, or PBS (10^6 pfu). Systemic TNF-α treatment was administered at days 4 and 6. *Data points*, mean tumor volumes; *bars*, SD. Tumors in the vvEMAP pretreated group (●, $n = 15$) showed significant regression and growth inhibition (day 7, $P < 0.05$ *versus* controls; days 8 and 9, $P < 0.01$; days 10–12, $P < 0.001$; and days 13 and 14, $P < 0.0001$). There was no significant difference between vvLuc-pretreated tumors (□, $n = 15$) and PBS treated controls (○, $n = 15$).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch189904668p.pdf by guest on 03 March 2024

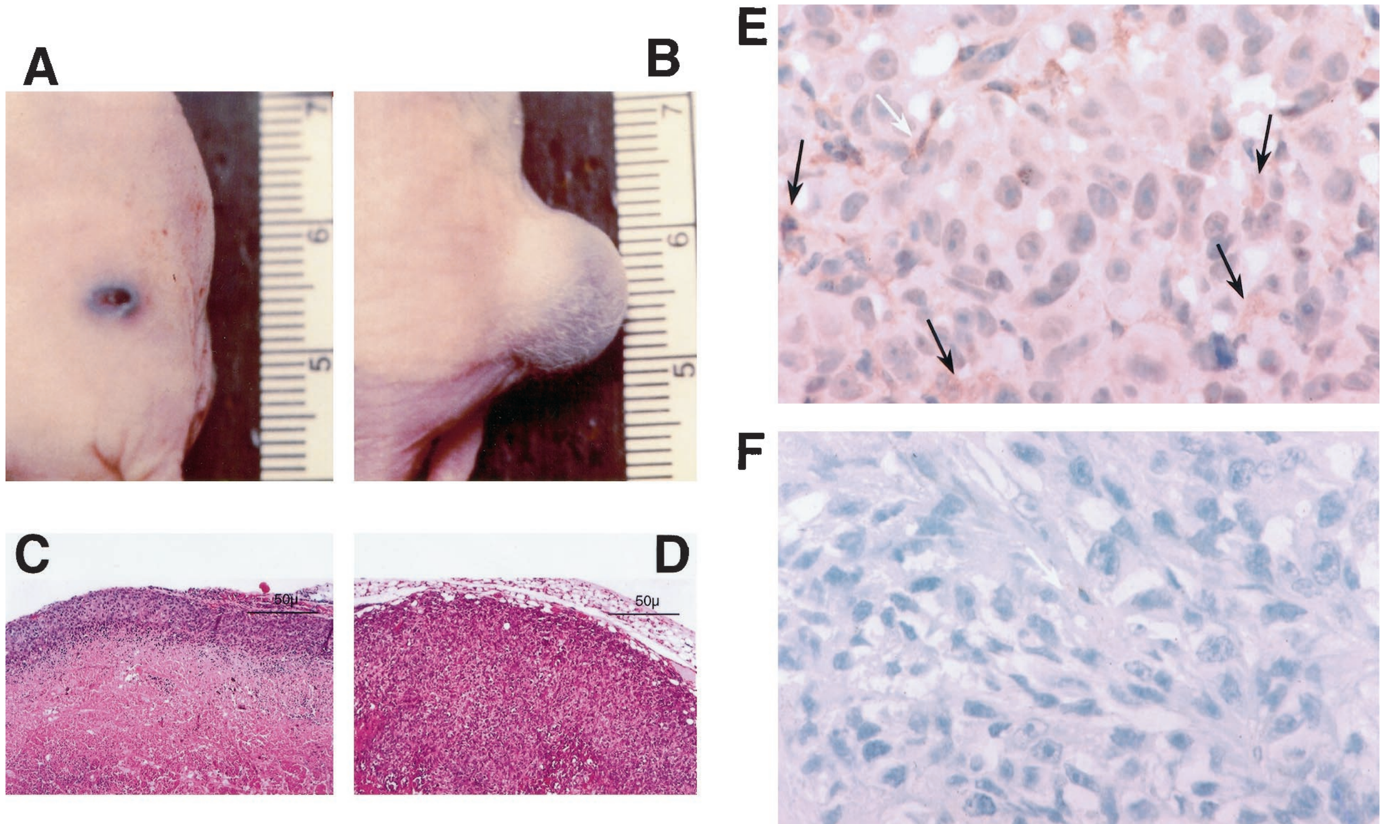

Fig. 4. Effects of TNF-α in previously TNF-resistant Pmel melanoma. *A*, macroscopic tumor regression and necrosis 14 days after systemic administration of vvEMAP. *B*, normal tumor growth observed after TNF treatment of animals pretreated with the control vector vvLuc. *C*, subtotal tumor necrosis, sparing only a small rim of viable tumor cells (magnification, 10×). *D*, nonnecrotic viable tumor in control animals. *E*, immunohistochemistry using anti-EMAP-II antibody demonstrates the presence of protein in vvEMAP-pretreated tumors. *Black arrows*, scattered staining in a predominantly membrane-bound and cytoplasmic pattern. *White arrows*, nonspecific melanin granula, which are also present in tumors from control animals (*F*).

(46). A tumor-specific sensitization gene therapy approach provides a unique opportunity to artificially render established tumors sensitive to the systemically administered TNF-α without increasing the vulnerability of healthy tissues and organs, thus facilitating systemic treatment with tolerable doses of the cytokine.

One important basis for the success of this approach is the unique ability of recombinant vaccinia viruses to specifically transfect tumors. It is not yet fully understood why systemic application of vaccinia viruses leads to preferential expression of the foreign gene in tumors (47). Whereas at least some gene expression can also be observed in other organs, the observed 100-fold increased expression of the genes transferred within tumors as compared with other tissues provides a window of opportunity for many treatment approaches, including the delivery of a "sensitizer" toward subsequent treatment, as demonstrated. Other approaches used in this context include delivery of genes encoding for prodrug converter enzymes in a so-called suicide gene therapy approach (48) and genes increasing the immunogenicity of tumors via expression or overexpression of antigens (49), costimulatory molecules (47, 50, 51), or cytokine gene transfer (52, 53).

Hypothetical reasons for the preferential gene expression within tumors include the relatively large size of the vector (>300 nm) in conjunction with the potentially leakier tumor neovasculature and a decreased immune clearance of the viral construct within tumors due to local immunosuppressive factors (54).

In our view, however, it is most likely that the insertion of the foreign genes into the locus of the viral *TK* gene, leading to the disruption of that gene, which plays a major role in the tumor specificity of the vector (55). Human tumor cells are metabolically much more active than benign tissue, providing an abundant pool of functional nucleotides that can compensate for the absence of the viral TK. Human melanoma, however, seems to be less permissive for vaccinia virus infection when compared directly to murine tumors (39). We have elsewhere demonstrated differences in gene expression of up to 4 orders of magnitude between tumors and other tissues when a syngeneic murine tumor is used in mice (39).

It is difficult to predict whether this tumor specificity will be preserved or even enhanced when the vector system is used in the clinical setting. Thus far, recombinant vaccinia viruses have mainly been used in Phase I clinical trials to induce specific immune responses against generalized tumors (56). The large amount of viral proteins of recombinant vaccinia viruses, particularly compared with other viral vectors, yields a massive immune response that induces long-lasting immunity against antigens encoded by the viral vectors (57). This, however, may limit the potential clinical use in a drug or cytokine-sensitizing gene therapy approach; repeated application of the same viral vector may likely lead to rapid destruction of the vector (58). At least transient replication and gene expression seem to be possible, even in preimmunized patients.

The high immunogenicity of the vector system also provides a potential safety benefit by virtually eliminating the likelihood of systemic infections with vaccinia virus in immunocompetent human hosts. In addition, although they are replication competent, the recombinant viral constructs we are using are severely attenuated by the disruption of their *TK* gene (59). Furthermore, the smallpox eradication program has established the safety of even intact vaccinia viruses

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch18990004668p.pdf by guest on 03 March 2024

(60). To date, there has been no vector-associated clinical toxicity reported in several Phase I cancer or other gene therapy trials using recombinant vaccinia viruses, whether administered intratumorally or i.v. (61, 62).

Although we were able to demonstrate dramatic responses and tumor regressions in our *in vivo* model, our sensitization/treatment concept did not eliminate all viable tumor cells, leading to eventual regrowth of responding tumors. Intervening earlier in the course of the disease may circumvent this problem. Furthermore, lowering the number of viable tumor cells below a certain threshold might even lead to complete clinical remissions and cures (63) because the host's natural antitumor activity is much more effective with limited tumor burden. There is, however, some indication that the recombinant vaccinia viruses that we use as vectors do not exert their tumor-tracking ability in an adjuvant setting, most likely because established tumors and tumor vasculature are needed, to some extent, for infection and amplification.

A previous attempt to sensitize human tumors toward TNF-α treatment by transfecting human tumor cells with the M_r 55,000 TNF receptor gene *in vitro* was reported recently (64). Although that attempt provided promising results, the concept has not yet been used for established nonsensitive tumors *in vivo*.

EMAP-II has been described as a tumor-derived cytokine initially by its ability to induce tissue factor procoagulant activity (65) and up-regulating the leukocyte adhesion molecules P-selectin and E-selectin. Its properties are multiple and have not yet been completely elucidated. (*a*) It has obvious proinflammatory effects and can be found in experimental autoimmune encephalomyelitis, neuritis, and uveitis (66). (*b*) It regulates attraction of monocytes/macrophages to the sites of inflammation (34). Recently, EMAP-II mRNA was found most abundant at sites of tissue remodeling in mouse embryos, suggesting that cells heading toward apoptotic cell death overexpress EMAP-II to attract macrophages for removal of dead cells (67). In fact, the release of mature EMAP-II was found coincident with apoptosis *in vitro* and *in vivo*.

Direct injection of recombinant EMAP-II leads to sensitization of TNF-α-resistant tumors (37). Conclusive evidence for the causal importance of EMAP-II expression for TNF-α response in human melanoma was provided by stable retroviral transduction of TNF-α resistant human melanoma cells with the EMAP-II gene *in vitro*, resulting in a phenotypic change of these tumors *in vivo*, rendering them susceptible to TNF-α treatment (35). The specific *in vivo* delivery of the gene into established non-EMAP-II-expressing TNF-α-resistant tumors and their subsequent regression and growth inhibition translates those findings into a clinically more realistic perspective because most tumors in patients will either express EMAP-II only at a very low level or not at all.

Although the exact mechanism of how EMAP-II sensitizes tumors toward TNF-α remains unclear, there is evidence that TNF receptor I is up-regulated in endothelial cells by EMAP-II in a dose-dependent fashion (68, 69), thus triggering death of endothelial cells in the presence of TNF-α and, subsequently, inducing ischemic tumor necrosis.

TNF-α exerts a very potent antitumor effect when the dose of the cytokine and the sensitivity of the targeted cells combine in a favorable manner. We have shown that modern gene transfer techniques provide us with the opportunity to artificially improve the local effectiveness of TNF-α in human melanoma while avoiding toxic side effects in other organs. Our results may lead to reevaluation of the role of TNF-α in clinical tumor therapy. The pretreatment of human tumors with recombinant vaccinia virus encoding for EMAP-II may sensitize those tumors toward TNF-α or at least increase the therapeutic window in moderately TNF-α-sensitive tumors, helping to reduce the necessary doses of the agent and, consequently, reduce toxicity and side effects.

ACKNOWLEDGMENTS

We thank Dr. Steven Rosenberg for helpful comments and Barbara Owen for technical assistance with the manuscript.

REFERENCES

1. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA, *72:* 3666–3670, 1975.
2. Fiers, W. Tumor necrosis factor: characterization at the molecular, cellular, and *in vivo* level. FEBS Lett., *285:* 199–212, 1992.
3. Prober, J. S. Effects of tumour necrosis factor and related cytokines on vascular endothelial cells. *In:* G. Bock and J. Marsh (eds.), Tumour Necrosis Factor and Related Cytotoxins, pp. 170–185. Chichester, United Kingdom: Wiley, 1984.
4. Clauss, M., Ryan, J., and Stern, D. Modulation of endothelial cell haemostasis properties by TNF: insights into the role of endothelium in the host response to inflammatory stimuli. *In:* D. Beutler (ed.), Tumour Necrosis Factors, The molecules and their emerging role in medicine, pp. 49–64. New York: Raven Press, 1992.
5. Corti, A., and Marcucci, F. Tumour necrosis factor: strategies for improving the therapeutic index. J. Drug Target, *5:* 403–413, 1998.
6. Hill, A. D., Redmond, H. P., Croke, D. T., Grace, P. A., and Bouchier-Hayes, D. Cytokine in tumour therapy. Br. J. Surg., *79:* 990–997, 1992.
7. Creaven, P. J., Brenner, D. E., Cowers, J. W., Huben, R. P., Wolf, R. M., Takita, H., Arbuck, S. G., Razack, M. S., and Proefrock, A. D. A Phase I clinical trial of recombinant human tumor necrosis factor given daily for 5 days. Cancer Chemother. Pharmacol., *23:* 186–191, 1989.
8. Budd, G. T., Green, S., Baker, L. H., Hersh, E. P., Weick, J. K., and Osborne, C. K. A Southwest oncology group Phase II trial of recombinant tumor necrosis factor in metastatic breast cancer. Cancer (Phila.), *68:* 1694–1695, 1991.
9. Orita, K., Fuchimoto, S., Murimoto, M., Ando, S., and Minowada, J. Early Phase II study of interferon-γ and tumor necrosis factor-α combination in patients with advanced cancer. Acta Med. Okayama, *46:* 103–112, 1992.
10. Tracey, K. J., Beutler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Milsark, I. W., Hariri, R. J., Fahey, T. J., III, Zentella, A., Albert, J. D., Shires, G. T., and Cerami, A. Shock and tissue injury induced by recombinant human cachectin. Science (Washington DC), *234:* 470–474, 1986.
11. Eggimann, P. C. R., Chassot, P-G., Liénard, D., Gerain, J., and Lejeune, F. Systemic and hemodynamic effects of recombinant tumor necrosis factor alpha in isolation perfusion of the limbs. Chest, *107:* 1074–1082, 1995.
12. Ksontini, R., MacKay, S. L., and Moldawer, L. L. Revisiting the role of tumor necrosis factor α and the response to surgical injury and inflammation. Arch. Surg., *133:* 558–567, 1998.
13. Fraker, D. L., Alexander, H. R., and Pass, H. I. Biologic therapy with TNF: systemic administration and isolation-perfusion. *In:* V. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Biologic Therapy of Cancer, pp. 329–345. Philadelphia: J. B. Lippincott, 1995.
14. Alexander, H. R., Bartlett, D. L., Libutti, S. K., Fraker, D. L., Moser, T., and Rosenberg, S. A. Isolated hepatic perfusion with tumor necrosis factor and melphalan for unresectable cancers confined to the liver. J. Clin. Oncol., *16:* 1479–1489, 1998.
15. Fraker, D. L., Alexander, H. R., Andrich, M., and Rosenberg, S. A. Treatment of patients with melanoma of the extremity using hyperthermic isolated limb perfusion with melphalan, tumor necrosis factor, and interferon-γ: results of a TNF dose escalation study. J. Clin. Oncol., *14:* 479–489, 1996.
16. Alexander, H. R., Bartlett, D. L., Fraker, D. L., and Libutti, S. K. Regional treatment strategies for unresectable primary or metastatic cancer confined to the liver. *In:* V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Cancer: Principles and Practice of Oncology, pp. 1–19. Philadelphia: J. B. Lippincott, 1996.
17. Bartlett, D. L., Ma, G., Alexander, H. R., Libutti, S. K., and Fraker, D. L. Isolated limb reperfusion with tumor necrosis factor and melphalan in patients with extremity melanoma after failure of isolated limb perfusion with chemotherapeutics. Cancer (Phila.), *80:* 2084–2090, 1997.
18. Eggermont, A. M., Schraffordt, K. H., Klausner, J. M., Schlag, P. M., Kroon, B. B., Ben-Air, G., and Lejeune, F. J. Isolated limb perfusion with high-dose tumor necrosis factor-α for locally advanced extremity soft tissue sarcomas. Cancer Treat. Res., *91:* 189–203, 1997.
19. Lejeune, F. J., Liénard, D., Leyvraz, S., and Mirimanoff, R. O. Regional therapy of melanoma. Eur. J. Cancer, *294:* 606–612, 1993.
20. Eggermont, A. M., Schraffordt, K. H., Klausner, J. M., Liénard, D., Kroon, B. B., Schlag, P. M., Ben-Air, G., and Lejeune, F. J. Isolation limb perfusion with tumor necrosis factor α and chemotherapy for advanced extremity soft tissue sarcomas. Semin. Oncol., *24:* 547–555, 1997.
21. Alexander, H. R., Bartlett, D. L., and Libutti, S. K. Isolated hepatic perfusion: a potentially effective treatment for patients with metastatic or primary cancers confined to the liver. Cancer J. Sci. Am., *4:* 2–11, 1998.
22. Nawroth, P., Handley, D., Matsueda, G., de Waal, R., Gerlach, H., Blohm, D., and Stern, D. Tumor necrosis factor/cachectin-induced intravascular fibrin formation in meth A fibrosarcomas. J. Exp. Med., *168:* 637–647, 1988.
23. Olieman, A. F. T., van Ginkel, R. J., Hoekstra, H. J., Mooyaart, E. L., Molenaar, W. M., and Koops, H. S. Angiographic response of locally advanced soft-tissue

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch18990004668p.pdf by guest on 03 March 2024

sarcoma following hyperthermic isolated limb perfusion with tumor necrosis factor. Ann. Surg. Oncol., *4:* 64–69, 1997.

24. Rosenberg, S. A. Gene therapy for cancer. J. Am. Med. Assoc., *268:* 2416–2419, 1992.
25. Rosenberg, S. A., Anderson, W. F., Blaese, M., Hwu, P., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Ettinghausen, S. E., Parkinson, D. N., and White, D. E. The development of gene therapy for the treatment of cancer. Ann. Surg., *218:* 455–464, 1993.
26. Nishihara, K., Barth, R. F., Wilkie, N., Lang, J. C., Oda, Y., Kikuchi, H., Everson, M. P., and Lotze, M. T. Increased in *vitro* and *in vivo* tumoricidal activity of a macrophage cell line genetically engineered to express IFN-γ, IL-4, IL-6, or TNF-α. Cancer Gene Ther., *2:* 113–124, 1995.
27. Hu, S. X., Ji, W., Zhou, Y., Logothetis, C., and Xu, H. J. Development of an adenovirus vector with tetracycline-regulatable human tumor necrosis factor α gene expression. Cancer Res., *57:* 3339–3343, 1997.
28. Stabe, M-J., Mauceri, H. J., Kufe, D. W., Hallahan, D. E., and Weichselbaum, R. R. Adenoviral TNF-α gene therapy and radiation damage tumor vasculature in a human malignant glioma xenograft. Gene Ther., *4:* 293–300, 1998.
29. Fushimi, K., Torigoe, K., Yamauchi, H., Furusako, S., Kurimoto, M., and Namba, M. Establishment of a human fibroblast cell line producing tumor necrosis factor (KMST-6/TNF) and growth inhibitory effects of its conditioned medium on malignant cells in culture. In Vitro Cell Dev. Biol. Anim., *34*: 463–467, 1998.
30. Oppenheim, J., and Fujiwara, H. The role of cytokines in cancer. Cytokine Growth Factor Rev., *7:* 279–288, 1996.
31. O'Rourke, M. G., Schmidt, C. W., O'Rourke, T. R., and Ellem, K. A. Immunotherapy, including gene therapy, for metastatic melanoma. Aust. N. Z. J. Surg., *67:* 834–841, 1997.
32. Marr, R. A., Hitt, M., Muller, W. J., Gauldie, J., and Graham, F. L. Tumour therapy in mice using adenovirus vectors expression human TNF. Int. J. Oncol., *12:* 509–515, 1998.
33. Bierhaus, A., Zhang, Y., Deng, Y., Mackman, N., Quehenberger, P., Haase, M., Luther, T., Muller, M., Bohrer, H., Greten, J., Martin, E., Baeverle, P. A., Wuldherr, R., Kisiel, W., Ziegler, R., Stern, D. M., and Nawroth, P. P. Mechanism of the tumor necrosis factor α-mediated induction of endothelial tissue factor. J. Biol. Chem., *270:* 26419–26432, 1995.
34. Kao, J., Ryan, J., Brett, G., Chen, J., Shen, H., Fan, Y-G., Godman, G., Familletti, P., Wang, F., Pan, Y-C., Stern, D., and Clauss, M. Endothelial monocyte-activating polypeptide II: a novel tumor-derived polypeptide that activates host-response mechanisms. J. Biol. Chem., *267:* 20239–20247, 1992.
35. Wu, P. C., Alexander, H. R., Huang, J., Hwu, P., Gnant, M., Berger, A. C., Turner, E., Wilson, O., and Libutti, S. K. *In vivo* sensitivity of human melanoma to tumor necrosis factor (TNF) is determined by tumor production of the novel cytokine endothelial-monocyte activating polypeptide II (EMAPII). Cancer Res., *59:* 205–212, 1998.
36. Tas, M., Houghton, J., Jakobsen, A., Tomachova, T., Carmichael, J., and Murray, J. Cloning and expression of human endothelial-monocyte-activating polypeptide 2 (EMAP-2) and identification of its putative precursor. Cytokine, *9:* 535–539, 1997.
37. Marvin, M., Libutti, S., Kayton, M., Kao, J., Hayward, J., Grikscheit, T., Fan, Y., Brett, J., Weinberg, A., Nowygrod, R., LoGerfo, P., Feind, C., Hansen, K., Schwartz, M., Stern, D., and Chabot, J. A novel tumor-derived mediator that sensitizes cytokine-resistant tumors to tumor necrosis factor. J. Surg. Res., *63:* 248–255, 1996.
38. Buller, R. M., Smith, G. L., Cremer, K., Notkins, A. L., and Moss, B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature (Lond.), *317:* 813–815, 1985.
39. Puhlmann, M., Brown, C. K., Gnant, M., Huang, J., Libutti, S. K., Alexander, H. R., and Bartlett, D. L. Vaccinia as a vector for tumor directed gene therapy: Biodistribution of a thymidine kinase deleted mutant. Cancer Gene Ther., in press, 1999.
40. Davison, A. J., and Moss, B. Structure of vaccinia virus early promoters. J. Mol. Biol., *210:* 749–769, 1989.
41. Davison, A. J., and Moss, B. Structure of vaccinia virus late promoters. J. Mol. Biol., *210:* 771–784, 1989.
42. Earl, P. L., and Moss, B. Expression of proteins in mammalian cells using vaccinia viral vectors. *In:* F. M. Ausubel, R. Kinston, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.), Current Protocols in Molecular Biology, pp. 16.15.1–16.18.10. New York: Greene/Wiley Interscience, 1991.
43. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin. An endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1997.
44. Balkwill, F. R. Tumour necrosis factor. Br. Med. Bull., *45:* 389–400, 1989.
45. Fiers, W. Biologic therapy with TNF: preclinical studies. *In:* V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Biologic Therapy of Cancer, pp. 295–327. Philadelphia: J. B. Lippincott, 1995.
46. Anonymous. The TNF-TIL Human Gene Therapy Clinical Protocol. Hum. Gene Ther., *1:* 441–480, 1990.
47. Peplinski, G. R., Tsung, K., Whitman, E. D., Meko, J. B., and Norton, J. A. Construction and expression in tumor cells of a recombinant vaccinia virus encoding human interleukin-1β. Ann. Surg. Oncol., *2:* 151–159, 1995.
48. Singhal, S., and Kaiser, L. R. Cancer chemotherapy using suicide genes. Surg. Oncol. Clin. N. Am., *7:* 505–536, 1998.
49. Bronte, V., Carroll, M. W., Goletz, T. J., Wang, M., Overwijk, W. W., Marincola, F., Rosenberg, S. A., Moss, B., and Restifo, N. P. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. Proc. Natl. Acad. Sci. USA, *94:* 3183–3188, 1997.
50. Hodge, J. W., Abrams, S., Schlom, K. J., and Kantor, J. A. Induction of antitumor immunity by recombinant vaccinia viruses expressing B7–1 or B7–2 costimulatory molecules. Cancer Res., *54:* 5552–5555, 1994.
51. Peplinski, G. R., Tsung, K., Casey, M. J., Meko, J. B., Fredrickson, T. N., Buller, R. M. L., and Norton, J. A. *In vivo* murine tumor gene delivery and expression by systemic recombinant vaccinia virus encoding interleukin-1β. Cancer J., *2:* 21–27, 1996.
52. Meko, J. B., Yim, J. H., Tsung, K., and Norton, J. A. High cytokine production and effective antitumor activity of a recombinant vaccinia virus encoding murine interleukin 12. Cancer Res., *55:* 4765–4770, 1995.
53. Qin, H., and Chatterjee, S. K. Cancer gene therapy using tumor cells infected with recombinant vaccinia virus expressing GM-CSF. Hum. Gene Ther., *7:* 1860, 1996.
54. Peplinski, G. R., Tsung, K., Meko, J. B., and Norton, J. A. *In vivo* gene therapy of a murine pancreas tumor with recombinant vaccinia virus encoding human interleukin-1β. Surgery (St. Louis), *118:* 185–191, 1995.
55. Peplinski, G. R., Tsung, K., and Norton, J. A. Vaccinia virus for human gene therapy. Surg. Oncol. Clin. N. Am., *7:* 575–588, 1998.
56. Kim, C. J., Cormier, J., Roden, M., Gritz, L., Mazzara, G. P., Fetsch, P., Hijazi, Y., Lee, K. H., Rosenberg, S. A., and Marincola, F. M. Use of recombinant poxviruses to stimulate anti-melanoma T cell reactivity. Ann. Surg. Oncol., *5:* 64–76, 1998.
57. Moss, B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc. Natl. Acad. Sci. USA, *93:* 11341–11348, 1996.
58. Paoletti, E. Applications of pox virus vectors to vaccination: an update. Proc. Natl. Acad. Sci. USA, *93:* 11349–11353, 1996.
59. Whitman, E. D., Tsung, K., Paxson, J., and Norton, J. A. *In vitro* and *in vivo* kinetics of recombinant vaccinia virus cancer-gene therapy. Surgery (St. Louis), *116:* 183–188, 1994.
60. Fenner, F., Henderson, D. A., Aritz, I., Jezek, Z., and Ladnyi, I. D. Smallpox and Its Eradication. Geneva: WHO, 1988.
61. McAneny, D., Ryan, C. A., Beazley, R. M., and Kaufman, H. L. Results of a Phase I trial of a recombinant vaccinia virus that expresses carcinoembryonic antigen in patients with advanced colorectal cancer. Ann. Surg. Oncol., *3:* 495–500, 1996.
62. Cooney, E. L., Collier, A. C., Greenberg, P. D., Coombs, R. W., Zarling, J., Arditti, D. E., Hoffman, M. C., Hu, S. L., and Corey, L. Safety of an immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. Lancet, *337:* 567–572, 1991.
63. Schabel, F. M., Jr. Concepts for systemic treatment of micrometastases. Cancer (Phila.), *35:* 15–24, 1975.
64. Sato, T., Yamauchi, N., Sasaki, H., Takahashi, M., Okamoto, T., Sakamaki, S., Watanabe, N., and Niitsu, Y. An apoptosis-inducing gene therapy for pancreatic cancer with a combination of 55-kDa tumor necrosis factor (TNF) receptor gene transfection and mutein TNF administration. Cancer Res., *58:* 1677–1683, 1998.
65. Clauss, M., Murray, J. C., Vianna, M., de Waal, R., Thurston, G., Nawroth, P., Gerlach, H., Gerlach, M., Bach, R., Familletti, P. C., and Stern, D. A polypeptide factor produced by fibrosarcoma cells that induces endothelial tissue factor and enhances the procoagulant response to tumor necrosis factor/cachectin. J. Biol. Chem., *265:* 7078–7083, 1990.
66. Schluesener, H., Seid, K., Zhao, Y., and Meyermann, R. Localization of endothelial-monocyte-activating polypeptide II (EMAP), a novel proinflammatory cytokine, to lesions of experimental autoimmune encephalomyelitis, neuritis and uveitis: expression by monocytes and activated microglial cells. Glia, *20:* 365–372, 1997.
67. Knies, U. E., Behrensdorf, H. A., Mitchell, C. A., Deutsch, U., Risau, W., Drexler, H. C. A., and Clauss, M. Regulation of endothelial monocyte-activating polypeptide II release by apoptosis. Proc. Natl. Acad. Sci. USA, *95:* 12322–12327, 1998.
68. Libutti, S., Vargas, H., Wu, P., Turner, E., Bartlett, D., Eliot, H., Brown, C., Wong, G., Stern, D., Fraker, D., and Alexander, H. R. Endothelial monocyte activating polypeptide II (EMAP II) induces expression of tumor necrosis factor (TNF) receptor P55 (R1) by endothelial cells. Proc. Soc. Surg. Oncol., *70:* 24, 1997.
69. Wu, P. C., Berger, A. C., Huang, J., Gnant, M., Turner, E., Alexander, H. R., and Libutti, S. K. *In vivo* tumors that overexpress endothelial monocyte-activating polypeptide II demonstrate upregulation of tumor necrosis factor receptor p55 on tumor neovasculature. Surg. Forum, *49*: 436–438, 1998.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/59/18/4668/3242555/ch18990468p.pdf by guest on 03 March 2024