Targeting tissue factor on tumor vascular endothelial cells and tumor cells for immunotherapy in mouse models of prostatic cancer

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The efficacy and safety of an immunoconjugate (icon) molecule, composed of a mutated mouse factor VII (mfVII) targeting domain and the Fc effector domain of an IgG1 Ig (mfVII/Fc icon), was tested with a severe combined immunodeficient (SCID) mouse model of human prostatic cancer and an immunocompetent mouse model of mouse prostatic cancer. The SCID mice were first injected s.c. with a human prostatic tumor line, forming a skin tumor that produces a high blood titer of prostate-specific antigen and metastasizes to bone. The icon was encoded in a replication-incompetent adenoviral vector that was injected directly into the skin tumor. The tumor cells infected by the vector synthesize and secrete the icon into the blood, and the blood-borne icon binds with high affinity and specificity to mouse tissue factor expressed on endothelial cells lining the lumen of the tumor vasculature and to human tissue factor expressed on the tumor cells. The Fc domain of the icon activates a cytolytic immune attack against cells that bind the icon. The immunotherapy tests in SCID mice demonstrated that intratumoral injections of the adenoviral vector encoding the mfVII/human Fc icon resulted in long-term regression of the injected human prostatic tumor and also of a distant uninjected tumor, without associated toxicity to the mice. Comparable results were obtained with a SCID mouse model of human melanoma. At the end of the experiments the mice appeared to be free of viable tumor cells. This protocol also could be efficacious for treating cancer patients who have vascularized tumors.

The tumor vasculature is a prime target for cancer therapy because of its essential role in the growth and survival of all solid tumors. One therapeutic strategy that targets the tumor vasculature involves inhibiting the growth of new blood vessels (angiogenesis) by administering an anti-angiogenic molecule such as endostatin (1). The rationale for this strategy is that angiogenesis occurs in growing tumors but not in normal adult tissues, except in special situations such as wound healing and pregnancy. Because a vasculature usually has formed by the time a tumor is detected, an anti-angiogenic strategy could prevent further growth of the vasculature but probably not eradicate the existing vasculature. We have developed a different therapeutic strategy for destroying the tumor vasculature, by targeting the endothelial cells lining the luminal surface of the vasculature with a molecule that can activate a cytolytic immune response against the targeted cells. Our choice of a specific target for the tumor vasculature is tissue factor (TF), a transmembrane receptor that forms an exceptionally strong and specific complex with its ligand, factor VII (fVII), as the initial step of the blood coagulation pathway (2). TF is not normally expressed on vascular endothelial cells (3, 4), but TF is expressed on endothelial cells of the tumor vasculature (5, 6). TF also is expressed on extravascular cells of several normal tissues and in the adventitial layer of the blood vessel wall (3, 4, 7). These cells are sequestered from contact with fVII by the tight endothelial cell layer of the normal vasculature (8), preventing blood clots from forming in normal tissues unless the endothelium is damaged. However, blood clots do occur in tumors (9, 10), presumably as a result of TF expression on tumor vascular endothelial cells and also on tumor cells that are accessible to fVII because the tumor vascular endothelium is leaky (8, 11–14). Although blood clots in tumors do not

prevent tumor growth and metastasis, otherwise cancer would not be a major cause of death, tumor growth has been inhibited by targeting additional TF molecules to the tumor vascular endothelium to enhance clotting (15).

In earlier reports we described an immunotherapy strategy for destroying the tumor vasculature by targeting TF on tumor vascular endothelial cells with an immunoconjugate (icon) molecule (16, 17). The model for the icon is a Camelid IgG1 antibody composed of two heavy chains without associated light chains, each heavy chain containing a V_H targeting domain conjugated directly to the Fc effector domain (18). The icon is similarly composed of two chains, each chain containing fVII as the targeting domain conjugated to a Fc effector domain (Fig. 1). The two fVII domains of the homodimeric icon provide an avidity effect that enhances binding to cells expressing multiple TF molecules (14). The Fc domain of the icon can activate a powerful cytolytic response against cells that bind the icon, mediated by components of the immune system containing Fc receptors, such as natural killer cells and the C1q protein that initiates the complement pathway (19). Because binding of the icon to TF in normal tissue could induce disseminated intravascular coagulation, a potentially lethal vascular disease, an amino acid substitution was introduced into the fVII domain of the icon to inhibit initiation of the coagulation pathway without reducing the strong affinity for TF.

The efficacy and safety for cancer immunotherapy of an icon composed of a mouse fVII domain and a human IgG1Fc domain (mfVII/hFc icon) was demonstrated in a severe combined immunodeficient (SCID) mouse model of human melanoma (17). In this model, melanoma cells are injected s.c. to generate a skin tumor or i.v. to generate metastatic lung tumors (20). To achieve continuous delivery of the icon to the systemic circulation, the icon was encoded in a replication-incompetent adenoviral vector that was injected intratumorally into a skin tumor. The infected tumor cells synthesize and secrete the icon, and the blood-borne icon molecules bind to TF on tumor vascular endothelial cells and tumor cells, resulting in regression of the injected skin tumor and uninjected lung tumors.

In the experiments reported here, we tested the efficacy and safety of the mfVII/hFc icon for immunotherapy of human prostatic cancer in a SCID mouse model. A human prostatic tumor cell line was injected s.c. to generate one or two skin tumors. One tumor was injected with the adenoviral vector encoding the icon or with a control adenoviral vector. In the mice injected with the control vector both the injected and uninjected tumors grew continuously,
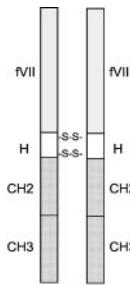
Fig. 1. Diagram of a fVII/Fc icon. fVII, fVII with a Lys$_{341}$ to Ala$_{341}$ mutation. H, Hinge region of an IgG1 Ig. CH2 and CH3, Constant regions in the Fc domain of an IgG1 Ig.

and the mice died or had to be euthanized within 63 days. In the mice injected with the vector encoding the icon both the injected and uninjected tumors regressed, and the mice appeared to be free of viable tumor cells when the experiments were terminated.

Materials and Methods

Cell Lines. C4–2 is a human prostatic cancer cell line derived from the LnCap cell line (21). RM-1 is a mouse prostatic cancer cell line. CHO is a Chinese hamster ovary cell line, and 293 is a human kidney cell line (ATCC CRL1573). TF2 is a melanoma cell line expresses a high level of TF (20). C4–2 cells were cultured in T4 medium (Sigma) + 10% FBS. RM-1 and 293 cells were cultured in DMEM + 10% FBS. CHO cells were cultured in RPMI1640 + 10% FBS.

Plasmid and Adenoviral Vectors for the Icons. The construction of the plasmid vectors for the mfVII/hFc and mfVII/mFc icons has been described (16, 17). The plasmid vector for the human fVII (hfVII)/hFc icon (GenBank accession no. 272774) was constructed as follows. The hfVII cDNA was amplified by PCR from a plasmid containing the hfVII cDNA; the 5′ primer was ACGATCTTA-AGCTTTGCAGAGATTTCATCATGGTCTCC, and the 3′ primer was ACGGTAACGGATCCGGGAAATGGGGCTCGC-AGGAGGAC. The amplified hfVII cDNA was cloned into the *Hin*dIII and *Bam*HI sites of the pcDNA3.1(+) vector (Invitrogen) in-frame with a cDNA encoding the human IgG1Fc domain. The mutation Lys$_{341}$ to Ala$_{341}$ was introduced into the hfVII cDNA by a mutagenesis procedure (Stratagene); the 5′ primer was GCAAG-GACTCCTGCGCGGGGGACAGTGGAGG, and the 3′ primer was CCTCCACTGTCCCCCGCGCAGGAGTCCTTGC.

The adenoviral vectors for the icons were constructed by using the AdEasy system as described (16, 17). The adenoviral vector particles (VP) titer was determined by diluting the adenoviral vector preparation 20× in 0.01% SDS/PBS and reading the absorbance at 260 nm; 1.0 OD = 1.0×10^{12} VP/ml.

Synthesis of Icon Protein in Transfected CHO Cells. The procedures for transfecting the pcDNA3.1(+) plasmid vectors for the icons into CHO cells and isolating stably transfected clones have been described (16, 17). The transfected CHO cells were cultured in serum-free CHO medium (EXCELL 301, JRH Biosciences, Lenexa, KS) supplemented with 1 μg/ml of vitamin K1 (Sigma). The icon protein was purified from the culture medium by affinity chromatography on Protein A beads (Pierce), and then concentrated and desalted by centrifugation through an Ultrafree-15 Biomax-50 filter (Millipore) in PBS or TBS buffer. The final concentration of the icon was determined with the Bio-Rad protein assay reagent.

Fluorescence-Activated Cell Sorting (FACS). Human melanoma cells (TF2) and mouse melanoma cells (B16F10) were harvested in 2 mM EDTA/PBS, washed and resuspended in FACS buffer (10 mM

Tris·HCl, pH 7.4/150 mM NaCl/10 mM CaCl$_2$/1% BSA/0.05% NaN$_3$) and kept on ice for 20 min. The cells were centrifuged, resuspended in 100 μl of a solution containing 10 μg/ml of mfVII/hFc or hfVII/hFc protein in FACS buffer, and incubated for 30 min at 37°C; the control cells were incubated in FACS buffer without added icon. After washing in FACS buffer, the cells were incubated for 30 min on ice with fluorescein-labeled anti-human IgGFc (Sigma) diluted 1:50 in FACS buffer. The cells were washed in PBS, resuspended in PBS with 2 μg/ml of prepidium idion, and analyzed on a Becton Dickinson FACsort instrument.

Mouse Models. The tumor cells for all experiments were grown as attached monolayer cultures, dissociated in 2 mM EDTA/PBS, centrifuged, washed, and resuspended in culture medium or PBS. For the human prostatic tumor experiments, female SCID/CB-17 mice 4–6 weeks old (Taconic Farms) were injected s.c. in one or both rear flanks with 1×10^6 C4–2 cells/mouse. The cells were suspended in 50 μl culture medium, and immediately before the injection 50 μl of Matrigel (Becton Dickinson) was added to the cell suspension. For the human melanoma experiments, 5×10^5 TF2 cells suspended in PBS were injected s.c. in both rear flanks of a SCID/CB-17 mouse. For the mouse prostatic tumor experiments, 4×10^6 RM1 cells suspended in PBS were injected s.c. in one rear flank of a female C57BL/6 mouse 4–6 weeks old. The schedules for subsequent intratumoral injections of the adenoviral vectors are described in the figure legends. To estimate the volume of a skin tumor, the exposed surface of the tumor was measured in two perpendicular directions, and the volume was calculated as (width)^2(length)/2.

Injection of the Adenoviral Vector into Mice. For each intratumoral injection into SCID mice, 50 μl of an adenoviral vector preparation was injected slowly into three or four sites in a skin tumor, being careful to prevent leakage of the solution from the tumor. For each i.v. injection into C57BL/6 mice, 50 μl of an adenoviral vector preparation was injected into a tail vein.

Icon Titer. A 96-well plate (Microfluor-2, Dynex Technologies, Chantilly, VA) was coated with 50 μl per well of a 20 μg/ml streptavidin solution in 0.05 M carbonate buffer, pH 9.6 for 2 h at 37°C and then overnight at 4°C. The coated wells were blocked with 2% BSA in PBS, incubated for 1 h at 37°C with 50 μl of a 20 μg/ml solution of biotinylated Protein A (Sigma) diluted in PBS, washed three times with PBS, and incubated at 37°C for 90 min with 50 μl of the mouse plasma samples or a purified mfVII/hFc icon standard. The wells were washed three times with PBS, incubated at 37°C for 1 h with 50 μl of 5 μg/ml goat anti-human IgGFc antibody (Vector) diluted in 2% horse serum, washed three times with PBS, incubated at 37°C for 1 h with 50 μl of 1 μg/ml fluorescein labeled rabbit anti-goat antibody conjugate (Vector) diluted in 1% BSA, washed three times with PBS, and 50 μl of PBS was added to each well. The fluorescence intensity was determined with excitation at 485/20 nm and emission at 530/25 nm and a gain of 40 on a multiwell plate reader (CytoFluoR II, Applied Biosystems). The icon titer was calculated from the mfVII/hFc icon standard curve.

Prostate-Specific Antigen (PSA). Blood samples were drawn from the eyes of the mice and mixed immediately with 0.128 M (3.8%) buffered sodium citrate (Becton Dickinson) at a ratio of 9:1 (vol/vol) blood/citrate. Plasma samples were prepared by centrifugation of the blood/citrate samples at maximum speed in a microfuge for 15 min at 4°C. The PSA assays were done in Leland Chung's laboratory at the University of Virginia, Charlottesville, as described (21).

Biodistribution of an Icon in SCID Mice. The mice received six injections of the adenoviral vector encoding the mfVII/hFc icon into a human melanoma skin tumor. Two days after the last
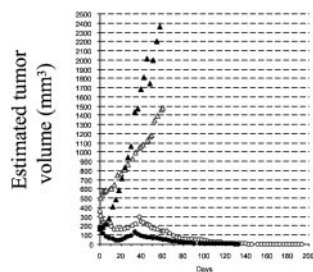
Fig. 2. Regression of a human prostatic tumor in SCID mice after intratumoral injections of an adenoviral vector encoding the mfVII/hFc icon. The first experiment involved two control mice (△) and two treated mice (○), and a second experiment involved five control mice (▲) and six treated mice (●). The mice were injected s.c. in one flank with the human prostatic cancer line C4–2. When the tumors had grown to an estimated volume of about 350 mm³ for the first experiment and about 180 mm³ for the second experiment, intratumoral injections were started (day 0). The dose/injection was 1×10^{10} VP. The mice received during the next 20 days a total of seven injections for the first experiment and six injections for the second experiment either of the vector encoding the mfVII/hFc icon or the control vector. The tumors injected with the control vector grew rapidly, causing the mice to become seriously ill; all of the mice died between days 49 and 63. The tumors injected with the vector encoding the icon regressed initially, but started to grow again. The mice then received three injections for the first experiment and four injections for the second experiment from days 33 to 45, after which the tumors regressed and did not grow again for the duration of the experiments. At the end of the experiments only minute necrotic nodules of tumor tissue remained, similar to the tumor section shown in Fig. 5. Each point is an average value that varied about ±20% among the mice.

injection, the mice were perfused to remove circulating icon, and the tumor, liver, and kidneys were dissected, fixed in formaldehyde, and sectioned for immunohistochemistry.

Prothrombin Time (PT). The PT assays were done in the Yale University Laboratory of Medicine on an automated instrument used for patient samples. The reaction cell contained 17 μl of purified icon in PBS and 34 μl of normal human plasma or fVII-depleted human plasma.

Results

Efficacy and Safety of a mfVII/hFc Icon for Immunotherapy of a Human Prostatic Tumor in SCID Mice.
In the first two experiments (Fig. 2), the mice were injected s.c. on one flank with cells from the human prostatic tumor line C4–2. When a tumor appeared on the skin, a series of intratumoral injections of the vector encoding the mfVII/hFc icon or of the control vector was administered during the

following 15 days. The tumors in the mice injected with the control vector continued to grow, and the mice either died or had to be euthanized by day 63. The tumors in the mice injected with the vector encoding the icon regressed initially, but then started to grow again. After the mice received three or four additional injections during days 33–45, the tumors again regressed and did not grow for the duration of the experiments. Only a small tissue mass consisting mainly of necrotic cells remained in these mice when the experiments were terminated.

The SCID mice were tested for blood levels of PSA, which is a sensitive measure of viable tumor cells. The PSA levels from days 49–63 in the control mice were relatively high as compared with the PSA levels on days 105 and 142 in the mice treated with the icon (Table 1). These data are consistent with the relative amounts of viable tumor tissue present in the control and icon-treated mice (Fig. 2).

The control and icon-treated mice were examined at necropsy for evidence of bleeding, clotting, or tumor metastasis. A bone tumor was present on the spine of one of the seven control mice, presumably derived by metastasis of the original skin tumor. None of the eight icon-treated mice had a metastatic tumor. In four of the control mice and four of the treated mice a rear ankle was swollen, possibly caused by an inflammatory reaction. No other morphological evidence of organ or tissue damage was detected in any of the mice. Histological sections of the brain, liver, lungs, kidneys, and spleen appeared normal.

The experiments in Fig. 2 demonstrated the efficacy of the mfVII/hFc icon against a tumor injected with the adenoviral vector encoding the icon. However, death from cancer usually occurs after metastasis and dissemination of a primary tumor, and most disseminated tumors probably would not be accessible for injection. Therefore, it is important to determine whether an icon produced by an injected tumor is efficacious against a tumor that has not been injected. The model used for this test involves generating skin tumors on both rear flanks of a mouse and injecting only one of the tumors either with the vector encoding the mfVII/hFc icon or with the control vector. This test was done for the human prostatic tumor (Fig. 3) and a human melanoma tumor (Fig. 5). In the mice injected with the control vector, the tumors on both flanks grew continuously, and the mice died or had to be euthanized by day 57. In the mice injected with the vector encoding the icon, the tumors on both sides regressed and did not grow again for at least several weeks after the last injection. At the end of the experiments, only a small mass of necrotic cells remained from both the injected and uninjected tumors (Fig. 4). No morphological abnormalities were detected in the mice at necropsy, and no histological abnormalities were detected in sections of the brain, liver, lungs, kidneys, and

Table 1. PSA titer in plasma of SCID mice after intratumoral injections of the adenoviral vector encoding the mfVII/hFc icon or the control adenoviral vector into a human prostatic tumor

Experiment	Control mice			Icon-treated mice		
	Injected mouse	Sample day	PSA, ng/ml	Injected mouse	Sample day	PSA, ng/ml
Fig. 2 no. 1	1	60	194.4 ± 6.3	1	142	0.6 ± 0.6
	2	49	8.0 ± 1.2*	2	142	0.6 ± 0.6
Fig. 2 no. 2	1	57	1,891 ± 10	1	105	0.6
	2	48	1,739 ± 37	2	105	0.0
	3	57	1,240	3	105	24
	4	63	170	4	105	0.0
	5	63	314			
Fig. 3	1	57	371	1	105	0.6
	2	57	1,504	2	105	0.4
	3	57	926 ± 33			
	Uninjected mouse		0.6 ± 0.6			

*The tumor in this mouse had extensive regions of necrotic cells, which could have accounted for the relatively low PSA titer.
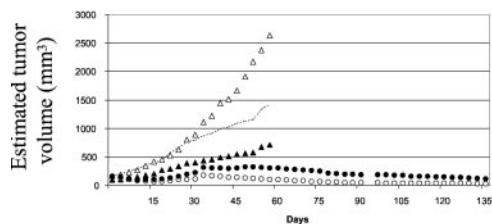
Fig. 3. Regression of two human prostatic tumors in SCID mice after intratumoral injections of an adenoviral vector encoding the mfVII/hFc icon into one tumor. The mice were injected s.c. in both rear flanks with the human prostatic cancer line C4–2. The resulting skin tumor on one flank was injected with the vector encoding the icon (four mice) or with the control vector (four mice) on days 0, 3, 6, 9, 12, 15, 33, 36, 39, and 42, whereas the tumor on the other flank was left uninjected. The dose was 1×10^{10} VP per injection. The control mice were euthanized on days 53–57 and the icon-treated mice on day 138. ○, Tumors injected with the vector encoding the icon. ●, Uninjected tumors in the icon-treated mice. △, Tumors injected with the control vector. ▲, Uninjected tumors in the control mice; the broken line shows the curve after adjusting each point for the difference between the estimated volumes on day 0 for the injected tumors (169 mm³) and uninjected tumors (88 mm³). Each point is an average value that varied about ±25% among the mice.

Fig. 5. Regression of two human melanoma tumors in SCID mice after intratumoral injections of an adenoviral vector encoding the mVII/hFc icon into one tumor. The mice were injected s.c. in both rear flanks with the human melanoma line TF2. The resulting skin tumor on one flank was injected with the vector encoding the icon (three mice) or with the control vector (three mice) on days 0, 3, 6, 9, 12, 15, 30, 36, 39, 42, and 45, whereas the tumor on the other flank was left uninjected. The dose was 6×10^{9} VP per injection. One control mouse was euthanized on day 53 and the other control mice on day 111. The icon-treated mice were euthanized on day 111. The histology of the melanoma tumors removed during necropsy was similar to the histology of the prostatic tumors shown in Fig. 4. ●, Tumors injected with the vector encoding the icon. ○, Uninjected tumors from the icon-treated mice. △, Tumors injected with the control vector. ▲, Uninjected tumors from the control mice. Each point is an average value that varied about ±20% among the mice.

spleen. These results demonstrate that the icon produced by a tumor injected with the adenoviral vector can activate a specific cytolytic immune attack not only against the injected tumor but also against a distant uninjected tumor.

Clearance of an Icon from the Blood of SCID Mice. The rate at which the mfVII/hFc icon is cleared from the blood of SCID mice was determined by injecting purified icon protein i.v. and monitoring the icon concentration in the plasma (Fig. 6A). The half-life of the icon was about 1 week, as compared with a half-life of about 3 weeks for a human IgG1 Ig and a half-life of about 4 h for hfVII. In SCID mice injected intratumorally with an adenoviral vector encoding the icon, the average icon concentration in the plasma did not decrease for at least 2 weeks after the last injection (Fig. 6B), presumably because the icon produced by the infected tumor cells replenishes the icon cleared from the blood.

Biodistribution of an Icon in SCID Mice. The biodistribution was determined after six intratumoral injections of the adenoviral vector encoding the mfVII/hFc icon into a human melanoma skin tumor. Two days after the last injection, the SCID mice were perfused with saline to remove unbound icon, and the icon remaining bound to the tumor, liver, and kidney was scanned by fluorescence immunohistochemistry (Fig. 7). The endothelium of the tumor vasculature showed intense fluorescence, in contrast to the liver and kidney that did not fluoresce. The failure to detect
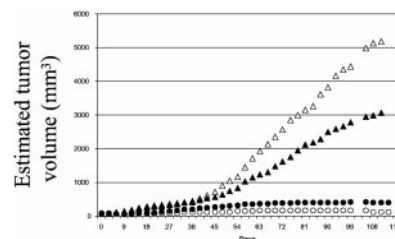
binding of the icon to the kidney is particularly significant, in view of the strong expression of TF by the glomeruli (3, 4). The immunohistochemical evidence is consistent with the morphological and histological evidence, cited above, showing that the icon causes damage to the tumor but not to normal tissues in icon-treated SCID mice.

Efficacy of a mfVII/mFc Icon in an Immunocompetent Mouse Model of Mouse Prostatic Cancer. C57BL/6 mice were injected s.c. on a rear flank with cells from the mouse prostatic cancer line RM-1. After a skin tumor had developed, a series of six i.v. injections of the vector encoding the mfVII/mFc icon or the control vector was administered during the following 15 days (Fig. 8). The injections were administered i.v. instead of intratumorally because the adenoviral vector does not infect the RM-1 tumor cells. The tumors in the mice injected with the control vector grew rapidly, and the mice had to be euthanized by day 13. Although the tumors in the mice injected with the vector encoding the icon grew at a significantly slower rate than in the control mice, the efficacy of the icon was weaker than in the SCID mouse experiments. Several differences between the immunocompetent and SCID mouse experiments, including the route of injection of the vector, the growth rate of the tumor and the capacity of the mice to generate a humoral and cellular immune response, could have contributed to the difference in the efficacy of the icon.
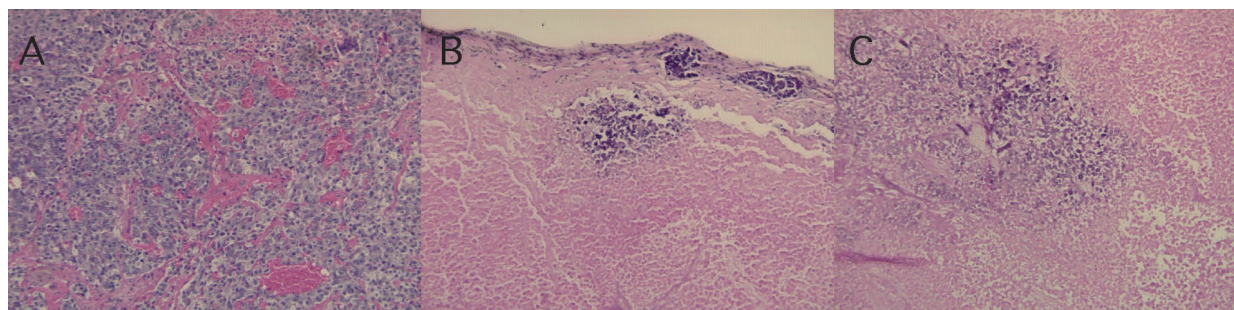
Fig. 4. Histology of the human prostatic tumors from the experiment described in Fig. 3. The tumors were excised during necropsy on day 138 for the icon-treated mice and on day 57 for the control mice. The sections were fixed in formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. (*A*) Tumor injected with the control vector, showing dense vascularization and viable tumor cells. (*B*) Injected tumor from the icon-treated mouse. (*C*) Uninjected tumor from the icon-treated mouse. Note that *B* and *C*, and also the areas not shown here, do not contain viable tumor cells. (Magnification: ×25.)

Fig. 6. Clearance of the mfVII/hFc icon from the plasma of SCID mice. (*A*) Five micrograms of icon protein was injected into the tail vein of two SCID mice on day 0, and blood samples were taken from the eyes on days 1, 3, 7, 14, 21, 32, 41, 47, and 51. The blood samples were mixed with buffered sodium citrate at a ratio of 1:9 (vol/vol), and the icon concentration was determined by fluorescence ELISA. (*B*) Five SCID mice were injected s.c. with 5×10^5 human melanoma TF2 cells per mouse. The resulting skin tumors were injected with the vector encoding the mfVII/hFc icon on days 0, 3, 6, 9, 12, and 15. The dose was 6×10^9 VP per injection. Blood samples were taken from the eyes on days 2, 17, 21, and 30, mixed with buffered sodium citrate at a ratio of 1:9 (vol/vol), and the icon concentration was determined by fluorescence ELISA.
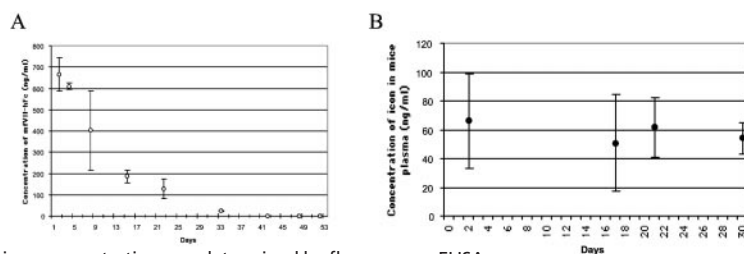
PT Tests for Bleeding and Clotting Induced by an Icon. Because the fVII domain in an icon has an amino acid substitution that inhibits initiation of coagulation, there is a risk that the icon might cause bleeding by competitively inhibiting the binding of endogenous fVII to TF. The risk of bleeding in a patient usually is assessed by a PT assay, which involves incubating a plasma sample from the patient with rabbit thromboplastin as a source of TF and measuring the time required for a clot to form. The plasma sample provides the essential clotting factors, including hfVII that complexes with rabbit TF to initiate the extrinsic blood coagulation pathway. The PT assay was used to determine the effect on the PT of adding the mfVII/hFc icon to the reaction. The PT remained normal at an icon concentration of 1 μg/ml and was prolonged by about 25% at a concentration of 5 μg/ml (Table 2), which is not sufficiently long to pose a significant risk of bleeding. In SCID mice injected intratumorally with the vector encoding the icon, the average icon concentration was about 0.06 μg/ml (Fig. 6*B*), far less than the concentration that would prolong the PT.

The PT assay also can be used to measure clotting activity of the icon by substituting fVII-depleted human plasma for complete human plasma. The PT without added icon was about 44 s, probably because of a small amount of hfVII remaining in the depleted plasma. Adding the mfVII/hFc icon decreased the PT to 25.8 s, indicating that the mutation in the fVII domain of the icon does not completely block its coagulation activity.
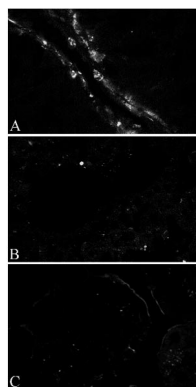
Preliminary Studies of the hfVII/hFc Icon. A clinical trial of efficacy and safety for the icon-based immunotherapy protocol would involve replacing the mfVII/hFc icon used for the experiments in SCID mice with a hfVII/hFc icon that contains a hfVII domain with the same substitution of alanine for Lys$_{341}$ as in the mfVII domain. The relative affinities of the mfVII/hFc and hfVII/hFc icons for binding to mouse TF and human TF were tested by FACS using mouse melanoma cells and human melanoma cells that express high levels of TF (Fig. 9). The displacement of the melanoma cells is greater with the mfVII/hFc icon than the hfVII/hFc icon, indicating that the mfVII/hFc icon binds with higher affinity than the hfVII/hFc icon to mouse TF and human TF. The FACS results are consistent with the results of coagulation tests, showing that mouse plasma has significantly higher coagulation activity than human plasma in the presence of human TF and mouse TF (22). Because of the extremely weak affinity of the hfVII/hFc icon for mouse TF, the efficacy of the icon could not be tested in the mouse models of cancer, which depend on the binding of the icon to mouse TF on tumor vascular endothelial cells.

The hfVII/hFc icon was tested for an effect on the PT by using complete human plasma (Table 2). The PT was not significantly prolonged by addition of up to10 μg/ml of the hfVII/hFc icon. The PT tests using fVII-depleted human serum show that the hfVII/hFc icon retains partial coagulation activity.

Discussion

The experiments reported here were designed principally to test the efficacy and safety of administering intratumoral injections of an adenoviral vector encoding the mfVII/hFc icon for immunotherapy of human prostatic cancer in SCID mice. Prostatic tumors were generated on one or two sides of the mice; the tumor on one side was injected with the vector whereas the tumor on the other side was left uninjected to serve as a model for disseminated metastatic cancer. The tumors were derived from the human prostatic cancer line C4–2, which produces a high titer of PSA and can metastasize to bone, similar to the properties of the disease in prostate cancer patients. In the control mice injected with a blank adenoviral vector, the tumors grew rapidly and the mice usually died within 63 days
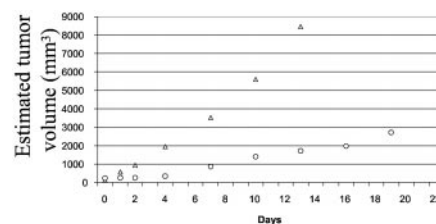

Fig. 7. Specific binding of the mfVII/hFc icon to the endothelium of a melanoma tumor in SCID mice. The mice were injected s.c. with the 6×10^9 VP of human melanoma cells TF2, and the resulting skin tumor was injected with the vector encoding the mfVII/hFc icon on days 0, 3, 6, 9, 12, and 15. On the second day after the last injection, the mice were euthanized and immediately perfused with 4% formaldehyde in PBS for 2 min. Sections of the tumor, liver, and kidney were postfixed for 1 h, washed with PBS, infused with 10% DMSO, and stored in liquid nitrogen. Frozen sections cut 6 μm thick were placed on lysine-coated slides, rehydrated in PBS, blocked with 1% BSA in normal goat serum for 1 h, and incubated with goat anti-human Fc antibody followed by tetramethylrhodamine B isothiocyanate-conjugated anti-goat antibody. The sections were coated with Vectashield antifade (Vector Laboratories) and photographed with fluorescent confocal optics. (*A*) Tumor. (*B*) Liver. (*C*) Kidney. The sections were prepared and photographed by T. Ardito in P. McPhaedron's laboratory, Department of Pathology at Yale University. (Magnification: ×375.)

Fig. 8. Effect of the mfVII/mFc icon on the growth of a mouse prostatic tumor in immunocompetent mice. The mouse prostatic tumor line *RM-1* was injected s.c. in C57BL/6 mice, and when a skin tumor had grown to the size indicated on day 0 the mice were i.v. injected with the vector encoding the mfVII/mFc icon (five mice) or with the control vector (five mice). The injections were done on days 0, 4, 7, 10, 13, and 16 with a dose of 1×10^{10} VP per injection. △, Control vector. ○, Vector encoding the icon. Each point is an average value that varied by about ±20% among the mice.

Hu and Garen

Table 2. Effect on the PT of the mfVII/hFc and hfVII/hFc icons

Icon concentration, µg/ml	hfVII/hFc icon		mfVII/hFc icon	
	Pooled normal plasma	FVII-deficient plasma	Pooled normal plasma	FVII-deficient plasma
0	11.8 ± 0.1	44.0 ± 0.2	11.8 ± 0.1	44.0 ± 0.2
1	11.7 ± 0.1	29.8 ± 12.5	11.7 ± 0.0	32.1 ± 0.0
5	11.5 ± 0.2	14.3 ± 0.3	14.2 ± 1.4	27.2 ± 0.1
10	12.0 ± 0.0	14.7 ± 0.1	14.6 ± 0.5	25.8 ± 0.0

Fig. 9. FACS assays for binding of the mfVII/hFc and hfVII/hFc icons to human TF on human melanoma cells (TF2) (*Left*) and mouse TF on mouse melanoma cells (B16F10) (*Right*). (*A*) Control cells not exposed to an icon. (*B*) Cells exposed to the hfVII/hFc icon. (*C*) Cells exposed to the mfVII/hFc icon. The relative displacement of each curve is proportional to the number of icon molecules bound to the cells.

after the tumors first appeared on the skin. In the mice injected with an adenoviral vector encoding the mfVII/hFc icon, virtually all of the viable tumor cells were eliminated and the mice appeared to be free of the disease and in good health at the end of the experiments, which lasted up to 194 days. The last injection of the vector occurred on day 53, demonstrating the long-term efficacy of the protocol.

Because death from cancer usually is associated with the metastatic disease, the clinical relevance of a protocol depends on the response of disseminated metastatic tumors, which might not be accessible for injection. The results of the experiments involving SCID mice with two tumors on opposite sides, which included human melanoma tumors in addition to human prostatic tumors, showed that injection of one tumor with the adenoviral vector encoding the icon caused regression of the uninjected tumor as well as the injected tumor. These results suggest that the protocol could be used to treat metastatic cancer patients who have at least one accessible tumor.

The major safety risk associated with the icon is bleeding that could result from binding of the icon to TF expressed on the vascular endothelium of normal tissues. The icon could then activate a cytolytic immune response against the normal vasculature or inhibit coagulation by competing with endogenous fVII for binding to TF. However, experiments in mice involving intratumoral injections of an adenoviral vector encoding an icon suggest that the risk of bleeding should not be significant. The relevant results reported here and in an earlier paper (17) are as follows. (*i*) The icon binds to the vascular endothelium of a human tumor in SCID mice, but does not bind to the mouse liver or kidney, and probably not to other normal tissues. (*ii*) Bleeding was not detected in the organs and tissues of the mice by morphological and histological examination. (*iii*) The icon concentration in the plasma
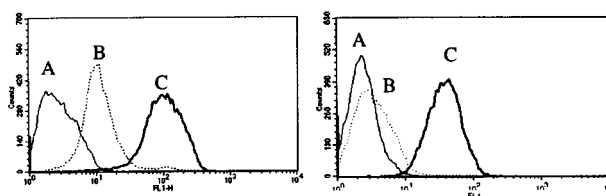
of the mice is about 1% of the minimum concentration that prolongs the PT, and therefore far below the concentration that might cause bleeding. (*iv*) In addition to the mouse data, extensive immunohistochemical screens of human tissues have shown that TF is not expressed on the vascular endothelium of normal tissues (3–6).

The impressive efficacy of the immunotherapy protocol in the mouse models of prostatic cancer and melanoma suggests that the protocol also could be efficacious for cancer patients. In a clinical trial the vector would encode a hfVII/hFc icon with the same amino acid substitution in the hfVII domain as in the mfVII domain of the icons used for the experiments in mice. The hfVII/hFc icon binds to human TF and should activate a cytolytic immune attack against the vasculature of human tumors. Because TF expression on tumor vascular endothelial cells probably occurs in all types of solid tumors, the protocol could have broad applicability for treating cancer patients. The protocol also might be applicable for treating patients suffering from other diseases associated with neovascularization, such as the "wet" exudative form of macular degeneration and diabetic retinopathy.

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