ABSTRACT An immunotherapy treatment for cancer that targets both the tumor vasculature and tumor cells has shown promising results in a severe combined immunodeficient mouse xenograft model of human melanoma. The treatment involves systemic delivery of an immunoconjugate molecule composed of a tumor-targeting domain conjugated to the Fc effector domain of human IgG1. The effector domain induces a cytolytic immune response against the targeted cells by natural killer cells and complement. Two types of targeting domains were used. One targeting domain is a human single-chain Fv molecule that binds to a chondroitin sulfate proteoglycan expressed on the surface of most human melanoma cells. Another targeting domain is factor VII (fVII), a zymogen that binds with high specificity and affinity to the transmembrane receptor tissue factor (TF) to initiate the blood coagulation cascade. TF is expressed by endothelial cells lining the tumor vasculature but not the normal vasculature, and also by many types of tumor cells including melanoma. Because the binding of an fVII immunoconjugate to TF might cause disseminated intravascular coagulation, the active site of fVII was mutated to inhibit coagulation without affecting the affinity for TF. The immunoconjugates were encoded as secreted molecules in a replication-defective adenovirus vector, which was injected into the tail vein of severe combined immunodeficient mice. The results demonstrate that a mutated fVII immunoconjugate, administered separately or together with a single-chain Fv immunoconjugate that binds to the tumor cells, can inhibit the growth or cause regression of an established human tumor xenograft. This procedure could be effective in treating a broad spectrum of human solid tumors that express TF on vascular endothelial cells and tumor cells.

An earlier study showed that immunoconjugates composed of an anti-human melanoma single-chain Fv (scFv) targeting domain, conjugated to the Fc region of human IgG1 as the effector domain, mediated specific lysis in vitro of human melanoma cells by natural killer cells and complement (1). The scFv molecules were isolated from a fusion-phage display library derived from the antibody repertoire of a melanoma patient who was vaccinated with autologous tumor cells (2, 3). The cognate antigen for the immunoconjugates is the melanoma-associated chondroitin sulfate proteoglycan MCSP, which is expressed predominantly on the surface of most melanoma cells (1, 4). The study reported here was designed to test further the therapeutic potential of an anti-MCSP scFv immunoconjugate in a severe combined immunodeficient (SCID) mouse xenograft model of human melanoma.

Also included in this study is another type of anti-tumor immunoconjugate containing as the targeting domain the zymogen factor VII (fVII), which binds with high affinity and specificity to the transmembrane receptor tissue factor (TF), and after activation initiates blood coagulation (5). TF is expressed by endothelial cells lining the vasculature of solid tumors but not of normal tissues (6, 7) and also is expressed by many types of tumor cells (8). Thus, TF provides a target on both the tumor vasculature and tumor cells for a fVII immunoconjugate. Binding of an fVII immunoconjugate to tumor vasculature endothelial cells should result in lysis of the endothelial cells and the loss of vascular functions essential for tumor growth and survival (9). In a human melanoma xenograft growing in SCID mice, the TF targets include human TF expressed by the tumor cells and mouse TF expressed by the endothelial cells in the tumor vasculature. Because mouse fVII (mfVII) binds strongly both to human TF and mouse TF, unlike human fVII that binds strongly to human TF but weakly to mouse TF (10), mfVII was chosen as the targeting domain for the fVII immunoconjugate. The complex formed between TF and fVII can result in disseminated intravascular coagulation (DIC), a potentially lethal complication associated with cancer (11). To prevent the possible occurrence of DIC in mice treated systemically with a fVII immunoconjugate, the active site of the targeting domain was mutated to inhibit initiation of the coagulation pathway without affecting the affinity for TF (12).

These two types of immunoconjugates, containing either an anti-MCSP scFv (G71–1) (3) or a mfVII active site mutant (mfVIIasm) as the tumor-targeting domain conjugated to the Fc region of human IgG1, were separately encoded in a replication-defective adenoviral vector (13), and the adenovirus was injected into the tail vein of SCID mice carrying a human melanoma xenograft. The cells infected by the adenovirus synthesized and secreted the encoded immunoconjugate into the blood for at least 1 week. The secreted immunoconjugates should be transported in the blood to the vasculature of the xenograft, where the mfVIIasm immunoconjugate can interact with the TF targets on the tumor vascular endothelial cells. Because the walls of the tumor vasculature are leaky (14), the immunoconjugates also should interact with the MCSP and TF targets on the melanoma cells. The Fc domain of the immunoconjugates should activate an immune response against the targeted tumor vascular endothelial cells and tumor cells by components of the immune system that remain functional in SCID mice, such as natural killer cells and complement. The results reported here demonstrate that the growth of an established human melanoma xenograft, expressing a low or high level of TF, can be inhibited by i.v. injections into the SCID mice of the adenoviral vectors encoding these immunoconjugates.

MATERIALS AND METHODS

Cell Lines. The melanoma cell lines LXSN, TF2, and LXSN/VEGF were derived from the human melanoma line

Abbreviations: scFv, single-chain Fv; MCSP, melanoma-associated chondroitin sulfate proteoglycan; SCID, severe combined immunodeficient; fVII, factor VII; mfVII, mouse fVII; mfVIIasm, mfVII active site mutant; TF, tissue factor; VEGF, vascular endothelial growth factor; CHO, Chinese hamster ovary.

*To whom reprint requests should be addressed.
YU-SITI by retroviral-mediated transfection and cloning (15). The LXSN line was transfected with the control retrovirus and expresses a low level of TF. The TF2 line was transfected with a retrovirus encoding TF cDNA and expresses a high level of TF. The LXSN/VEGF line was transfected with a retrovirus encoding vascular endothelial growth factor (VEGF) cDNA and expresses high level of VEGF. The human kidney line 293 was purchased from the American Type Culture Collection.

**Plasmid Vector.** The construction of the plasmid encoding the scFv (G71–I) immunoconjugate has been described (1). For the construction of the vector encoding the mFII immunoconjugate, the mFII cDNA was amplified by PCR from a mouse liver cDNA library (Quick-Clone cDNA, CLONTECH) using the 5′ primer ACAGTCTTAAGCT- TCCCCACAGTCTCATGTGGTCCAA and the 3′ primer ACAGTAAACGGATCCCCGATGTTGGAGTCGGAAA- CCCC (16). The amplified mFII cDNA, which contains the leader and coding sequences without a stop codon, was cloned into the HindIII and BamHI sites of the pCNA3.1 (+) vector (Invitrogen) in-frame with a cDNA encoding the human IgG1 Fc domain (1). The vector DNA was amplified in HB101 competent cells (Life Technologies, Grand Island, NY) and sequenced. The active site of mFII cDNA was mutated by substituting an alanine codon for Lys-341 for Lys (12). The mutagenesis procedure was done as described in the QuickChange site-directed mutagenesis manual (Stratagene). The 5′ primer was GGTACCAAGGACCCCTGCGGGTGAACGCCTGG- TGCCCCCA, and the 3′ primer was TGGGCCCCAGCTGT- CACCCGGCGCCGGTCTTTGGATC. The mFII cDNA with the active site mutation is designated mFIIasm. The plasmid containing mFIIasm cDNA was transformed into HB101 competent cells, and transformed colonies were selected on 2×TY/carbenicillin agar. The sequence of the plasmid DNA showed a substitution of an alanine codon (GCA) for Lys-341 codon (AAG) in the plasmid DNA showed a substitution of an alanine codon (GCA) for Lys-341 codon (AAG) in the pCNA3.1 (+) vector in the plasmid DNA showed a substitution of an alanine codon (GCA) for Lys-341 codon (AAG) in the plasmid DNA showed a substitution of an alanine codon (GCA) for Lys-341 codon (AAG) in the plasmid DNA.

**Synthesis of Immunconjugates in Chinese Hamster Ovary (CHO) Cells.** The procedures for transfecting the immunoconjugate cDNAs into CHO cells and isolating clones were described (1). For the construction of the CHO cells, the plasmid containing mFIIasm cDNA was transformed into HB101 competent cells, and transformed colonies were selected on 2×TY/carbenicillin agar. The sequence of the plasmid DNA showed a substitution of an alanine codon (GCA) for Lys-341 codon (AAG) in the mFIIasm cDNA.

**Immunohistochemistry.** Paraffin sections of the tumors and organs were incubated in PBS + 0.3% H2O2 for 30 min and blocked in TBS/BSA buffer for 30 min. A solution containing 10 µg/ml of the mFIIasm immunoconjugate in TBS/BSA/Ca2+ buffer, or as a control the buffer without the immunoconjugate, was added to the sections and incubated at 37°C for 1 hr. After washing three times in the same buffer, the sections were incubated at room temperature for 1 hr with anti-human with NotI. The immunoconjugate cDNAs were ligated into the shuttle vectors by incubation with T4 DNA ligase at 16°C overnight, and the shuttle vectors were transformed into HB101 competent cells by heat shock. In the transformation procedure, the shuttle vectors and pAdTrack-CMV DNAs were digested with PmeI at 37°C for 2 hr. A mixture of 500 ng shuttle vector DNA and 100 ng pAdEasy-1 DNA was electroporated into BJ5183 competent cells, and the cells were shaken at 37°C for 15 min and plated on LB/kanamycin agar. The plates were incubated at 37°C overnight, and transformed colonies were isolated. The plasmid DNAs were purified from minipreps and screened for recombinant adenoviral DNA by electrophoresis on 0.6% agarose gels.

The recombinant adenoviral DNAs encoding the immunoconjugates were transfected into LXSN cells, following the protocol described above for transfecting CHO cells. The cells were collected 7 days after transfection, and the adenoviruses were released by three freeze-thaw cycles and amplified by infecting 293 cells in one 150-mm culture plate. After 2 days, the adenoviruses were harvested as described above and amplified again by infecting 293 cells in 20 culture plates. The amplified adenoviruses were harvested 2 days later and purified by centrifugation in CsCl. The final yields usually were about 1013 virus particles as estimated from the absorbance at 260 nm; the conversion is 1 OD unit = 1 × 1012 particles. The purified adenoviruses were dialyzed against PBS and stored at −80°C.

**SCID Mice Experiments.** All animal protocols were approved by the Yale Institutional Committee. The SCID mice were 4- to 5-week-old females from Taconic Farms. The mice were injected s.c. into the right rear flank with 5 × 106 TF2 or LXSN human melanoma cells. After the tumors had grown to a palpable size below the skin surface (~5 mm3) or to a larger size above the skin surface (~50 mm3), the mice were injected via the tail vein with the adenoviral vector encoding an immunoconjugate, or as a control with the adenoviral vector that does not encode an immunoconjugate. The concentration of immunoconjugate protein secreted into blood was measured by collecting about 0.1 ml of blood from one eye into a microcapillary tube coated with heparin and centrifuging the blood to remove cells. The supernatant plasma was diluted with sodium bicarbonate buffer, pH 9.6 and distributed into wells of probind assay plates (Falcon), and the plates were incubated first at 37°C for 2 hr and then at 4°C overnight. The wells were blocked with 5% nonfat milk in PBS for 30 min and washed three times with PBS, and a peroxidase-labeled anti-human IgG antibody diluted 1:2,000 in 5% nonfat milk was added to the wells. The plates were incubated for 1 hr at room temperature and washed in PBS, and the peroxidase substrate OPD was added and absorbance was measured at 490 nm in a microplate reader. The protein standard was human IgG (Sigma), which we purified by chromatography on Protein A beads.

The size of a tumor appearing on the skin of a SCID mouse was measured in two dimensions with a caliper, and the tumor volume was estimated by the formula (width)2 (length)/2. At the end of an experiment, the mice were dissected, and the tumors were weighed. The organs were examined for morphological evidence of damage, and paraffin sections were prepared for histological examination.
γ-chain antibody labeled with alkaline phosphatase, stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, which produces a blue color, and counterstained with methyl green.

RESULTS

Properties of the Immunoconjugates. The scFv (G71–1) and the mfvIIasm immunoconjugates were synthesized in CHO cells and purified from the culture medium by affinity chromatography on Protein A beads. An earlier analysis by SDS-PAGE showed that the G71–1 immunoconjugate is composed of two identical chains, presumably coupled by disulfide bridges between the hinge regions of the Fc domains (1). The same result was obtained with the mfvIIasm immunoconjugate (data not shown). Because the mfvIIasm immunoconjugate has two targeting domains, as compared with the single targeting domain in the monomeric endogenous FVII molecule, it can bind cooperatively to two TF molecules, resulting in stronger binding than endogenous FVII to cells expressing TF. A competitive fluorescence-activated cell sorting assay (Fig. 1) showed that human FVIIa competes on an equimolar basis with the mfvIIasm immunoconjugate for binding to half of the accessible sites on human melanoma cells, probably because only one of the targeting domains on the immunoconjugate molecule can bind to TF at these sites. The binding of the mfvIIasm immunoconjugate to the remaining sites could not be competed in the presence of a 10-fold excess of human FVIIa, suggesting that both targeting domains of the immunoconjugate molecule can bind at these sites and provide a strong avidity effect. It appears that only about half of the TF molecules on the melanoma cells are sufficiently close to a second TF molecule to form a cooperative binding site for both targeting domains on a mfvIIasm immunoconjugate.

The xenografts for the immunotherapy tests were generated from the human melanoma lines LXSN and TF2, which express, respectively, low or high levels of TF. The mfvIIasm immunoconjugate binds more extensively to the TF2 cells than to the LXSN cells as determined by fluorescence-activated cell sorting (Fig. 2), consistent with the higher level of TF expression by the melanoma cells. (Fig. 2) showed that human FVIIa competes on an equimolar basis with the mfvIIasm immunoconjugate for binding to half of the accessible sites on human melanoma cells, probably because only one of the targeting domains on the immunoconjugate molecule can bind to TF at these sites. The binding of the mfvIIasm immunoconjugate to the remaining sites could not be competed in the presence of a 10-fold excess of human FVIIa, suggesting that both targeting domains of the immunoconjugate molecule can bind at these sites and provide a strong avidity effect. It appears that only about half of the TF molecules on the melanoma cells are sufficiently close to a second TF molecule to form a cooperative binding site for both targeting domains on a mfvIIasm immunoconjugate.

For systemic delivery to SCID mice, each immunoconjugate was encoded as a secreted molecule in the replication-defective adenoviral vector system based on pAdEasy-1 (13), and the vectors were injected into the tail vein of mice that had first been injected s.c. with human melanoma cells. The initial immunotherapy tests involved injecting each vector separately, and both vectors together, into the mice that had developed a palpable TF2 xenograft. A total of three injections were administered at weekly intervals, and the experiment was terminated 6 days after the last injection. The concentration of the immunoconjugates in the blood was monitored by ELISA after the first and second injections (Fig. 4). The average concentration after the first injection was 4 mg/ml for the G71–1 immunoconjugate and 0.04 mg/ml for the mfvIIasm immunoconjugate, indicating that the rate of synthesis was about 100-fold higher for the G71–1 immunoconjugate than for the mfvIIasm immunoconjugate. The concentration of each immunoconjugate increased after the second injections, indicating that additional cells had been infected by the adenoviruses. The growth of the xenografts was monitored by measuring in two dimensions the size of the tumor appearing on the skin surface, and by using the measurements to estimate the tumor volume (Fig. 5). In the control mice injected with the adenovirus that does not encode an immunoconjugate, the tumor grew continuously at a relatively fast rate, reaching an average volume of about 2,000 mm³ after 20 days. In the mice injected with an adenovirus encoding an immunoconjugate, tumor growth was inhibited; the inhibition was stronger for the mfvIIasm immunoconjugate than for the G71–1 immunoconjugate. All of the mice remained active and appeared healthy at the end of the experiment, and histological examination of the liver, spleen, lung, kidney, and brain did not show any evidence of necrosis, clotting, or bleeding (data not shown). However, many of the liver cells were enlarged, probably because the adenoviral vectors infect mainly liver cells (18), which continuously synthesize high levels of the encoded immunoconjugates. The tumor weights after autopsy were lower in the mice treated with the immunoconjugates than in the control mice, consistent with the estimated tumor volumes (Fig. 6). The strongest reduction of tumor weight occurred in the mice treated with both immunoconjugates.

The next two experiments were designed to test two parameters that could affect the therapeutic efficacy of the immunoconjugates, namely the initial size of the xenograft and the level of TF expression by the melanoma cells. (i) The preceding immunotherapy tests involved palpable melanoma xenografts that had grown to an estimated volume of about 5 mm³,
corresponding to a small tumor in humans. To test the therapeutic efficacy of the immunoconjugates against a larger xenograft, TF2 xenografts were allowed to grow to an estimated volume of about 50 mm³ before starting tail vein injections of the two adenoviral vectors. The mice received four injections during a period of 3 weeks, and the experiment was terminated 2 days after the last injection. The average tumor volume in the mice injected with the adenoviruses encoding the immunoconjugates was about the same at the end as at the start of the experiment, in contrast to the average tumor volume in the mice injected with the control adenovirus, which increased by a factor of about 27 during the same period (Fig. 7). These results show that tumor growth is inhibited as effectively with the larger tumor as with the smaller tumor. One of the five mice injected with the adenovirus encoding the immunoconjugates died 5 days after the third injection; the cause of death could not be determined because the mouse was not recovered in time for examination. (ii) A parameter that might affect the efficacy of the mfVIIasm immunoconjugate is the level of TF expression, which varies among different tumors (8). To study the effect of varying the expression of TF by the melanoma cells in a xenograft, the melanoma line LXSN was used to generate a xenograft expressing a low level of TF, for comparison with the xenograft generated from the related line TF2, which expresses a higher level of TF (15). After the xenografts reached a palpable size, the mice received during the next 3 weeks five injections of the adenovirus encoding the mfVIIasm immunoconjugate or the control adenovirus (Fig. 8). In the five mice injected with the control adenovirus the xenograft grew continuously, the average volume increasing to 1,350 mm³ on the second day after the last injection. During the same period the average volume of the xenografts in the mice injected with the mfVIIasm immunoconjugate increased to 20 mm³, indicating that the inhibition of tumor development is comparable for the LXSN and TF2 xenografts (compare Figs. 5 and 8). The autopsies performed 1 day after the last injection showed that the xenograft had been eradicated in two of the five mice injected with the adenovirus encoding the mfVIIasm immunoconjugate; the average tumor weight in the other three mice was 0.11 g as compared with the average weight of 0.75 g in the five mice injected with the control adenovirus. The small tumors recovered from these three mice showed extensive regions of cell necrosis, which did not occur in the larger tumors from the control mice (Fig. 9). All of the mice appeared healthy at the end of this experiment, but a morphological examination of the dissected mice revealed damage to the liver and spleen in the five mice injected with the adenovirus encoding the mfVIIasm immunoconjugate. Histological examination of the liver and spleen showed that many of the liver cells were enlarged, and the spleen was extensively infiltrated with erythrocytes. Enlarged liver cells also occurred in a previous experiment after three injections of the adenovirus encoding the mfVIIasm immunoconjugate, but the spleen was normal, indicating that the defects in the spleen developed in the course of the last two injections. One of the mice also had a subdural brain hemorrhage, which did not occur in other mice from this experiment or any of the previous experiments. It is uncertain whether this defect was induced by the binding of the mfVIIasm immunoconjugate to the vasculature and tumor cells. (C) Immunohistochemical control without the mfVIIasm immunoconjugate. Magnification: ×85.
A SCID mouse xenograft model of human melanoma was used to test the therapeutic potential of an immunotherapy procedure designed to target both the tumor vasculature endothelial cells and tumor cells for cytolysis by the host immune system. The procedure involved systemic delivery to SCID mice of two immunooconjugates, each composed of a tumor-targeting domain conjugated to the Fc region of a human IgG1 heavy chain, forming a homodimeric molecule similar to a Cameld heavy-chain antibody (19). For one type of immunooconjugate, the tumor-targeting domain was the human scFv molecule G71–1 that binds to the melanoma antigen MCSP (1, 4) expressed by the melanoma cells in the xenografts. For the other type of immunooconjugate, the tumor-targeting domain was a mfVII molecule that binds specifically and tightly to TF, both to mouse TF expressed by the tumor vasculature endothelial cells and to human TF expressed by the melanoma cells in the xenografts. To decrease the risk of disseminated intravascular coagulation that might result from the binding of a IV VIIa coagulation pathway, an active site mutation was introduced into the mfVII targeting domain (mfVIIasm), inhibiting the proteolytic activity required to initiate the blood coagulation pathway.

An earlier in vitro study showed that the G71–1 immunooconjugate mediates cytolysis of cultured human melanoma cells by natural killer (NK) cells and complement (1). Because SCID mice retain the capacity to produce functional NK cells and complement, the immunooconjugates also could mediate cytolysis of the targeted tumor cells and vascular endothelial cells of a human melanoma xenograft growing in SCID mice. Systemic delivery of the immunooconjugates to SCID mice was achieved by tail vein injections of a replication-defective adenoviral vector encoding the immunooconjugates, which were secreted into the blood for at least 1 week after each injection. The mice first were injected s.c. with a human melanoma cell line that expresses either a low or high level of TF, and the resulting xenograft was allowed to grow into a palpable size (day 0). Five mice were injected with 9 × 10^11 adenoviruses encoding the mfVIIasm immunooconjugate, and five mice were injected with 4 × 10^11 control adenoviruses. Additional injections were done on days 7, 13, 21, and 24, and on day 25 the mice were dissected for morphological and histochemical examination. The estimated tumor volumes are the averages for the five mice in each group.
adenoviral vectors, or by using a different type of vector. Another possible option is to administer the immunoconjugates directly as proteins.

Although the immunoconjugate concentration in the blood of SCID mice injected with an adenoviral vector was about 100-fold higher for the $G71-1$ immunoconjugate than for the $mfVIIasm$ immunoconjugate, the inhibitory effect on a human melanoma xenograft nevertheless was stronger with the $mfVIIasm$ immunoconjugate. A key advantage of the $mfVIIasm$ immunoconjugate is the binding that occurs to tumor vascular endothelial cells as well as to tumor cells, in contrast to the $G71-1$ immunoconjugate that binds only to melanoma cells. The binding to the tumor vasculature should be tumor specific, because TF is not expressed by the normal vasculature. Although TF is expressed by several other normal tissues, such as brain, lung, and kidney glomeruli, these TF molecules are not accessible to endogenous fVII or aVII immunoconjugate because the blood vessel walls form a barrier separating larger blood components from adjacent cells. However, tumor blood vessels are leaky (14), allowing access to TF expressed by tumor cells. Thus, a human $fVIIasm$ immunoconjugate could be an effective therapeutic agent for a broad spectrum of human tumors expressing TF on the vascular endothelial cells and tumor cells. The therapeutic efficacy of a human $fVIIasm$ immunoconjugate could be enhanced by also administering a human scFv immunoconjugate that binds to a tumor target other than TF.

In considering a clinical test of the immunoconjugates, which might require maintaining an adequate titer in the patient’s blood for a prolonged period, an immune rejection response to the immunoconjugates and/or the adenoviral vector could be a potential obstacle. Because the tumor-targeting and Fc effector domains of the immunoconjugates are derived from human sources for clinical protocols, the immunoconjugates should be tolerated by the human immune system. Although it was possible to use the adenoviral delivery system for repeated injections in SCID mice, an adenovirus might be too immunogenic in patients for this purpose. To avoid immune rejection of the vector, a nonimmunogenic vector could be substituted for the adenovirus, or the immunoconjugates could be administered directly as proteins.

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![Figure 9](Image)

**Fig. 9.** Histochemistry of the LXSN xenografts from the experiment reported in Fig. 8. The xenografts were dissected on day 25 and embedded in paraffin, and sections were stained with hematoxylin + eosin. (A) Xenograft from a control mouse injected with the adenovirus that does not encode an immunoconjugate. (B) Xenograft from a mouse injected with the adenovirus encoding the $mfVIIasm$ immunoconjugate. Magnification: ×245.