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Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model

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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 a 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 predominately 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 a 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 xenograph, 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

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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.

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YU-SIT1 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 vector encoding the scFv (*G71–1*) immunoconjugate has been described (1). For the construction of the vector encoding the mfVII immunoconjugate, the mfVII cDNA was amplified by PCR from a mouse liver cDNA library (Quick-Clone cDNA, CLONTECH) by using the 5′ primer ACGATCTTAAGCTTCCCCACAGTCTCATCATGGTTCCA and the 3′ primer ACGGTAACGGATCCCAGTAGTGGGAGTCGGAAAACCCC (16). The amplified mfVII cDNA, which contains the leader and coding sequences without a stop codon, was cloned into the *Hin*dIII and *Bam*HI sites of the pcDNA3.1(+) 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 mfVII cDNA was mutated by substituting an alanine codon for Lys-341 (12). The mutagenesis procedure was done as described in the QuickChange site-directed mutagenesis manual (Stratagene). The 5′ primer was GGTACCAAGGACGCCTGCGCGGGTGACAGCGGTGGCCCA, and the 3′ primer was TGGGCCACCGCTGTCACCCGCGCAGGCGTCCTTGGTACC. The mfVII cDNA with the active site mutation is designated *mfVIIasm*. The plasmid containing *mfVIIasm* 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 (GCG) for the Lys-341 codon (AAG) in the *mfVIIasm* DNA.

Synthesis of Immunoconjugates in Chinese Hamster Ovary (CHO) Cells. The procedures for transfecting the immunoconjugate cDNAs into CHO cells and isolating clones were described (1). The transfected CHO cells were cultured in CHO serum-free medium (EX-CELL 301, JRH Biosciences, Lenexa, KS); for synthesis of the *mfVIIasm* immunoconjugate, the CHO serum-free medium was supplemented with vitamin K1 (Sigma) to a final concentration of 1 μg/ml (17). The immunoconjugates were purified by affinity chromatography on Protein A beads (Pierce) and were concentrated and desalted by centrifugation through an Ultrafree-15 Biomax-50 filter (Millipore) and adjusted to 10 mM Tris·HCl, pH 8.0. The immunoconjugate concentrations were measured by the Bio-Rad protein assay procedure.

Fluorescence-Activated Cell Sorting. Melanoma cells were harvested in nonenzymatic dissociation solution (Sigma), washed and resuspended in TBS/BSA/Ca^{2+} (10 mM Tris·HCl, pH 7.4/150 mM NaCl/20 mM $CaCl_2$/1% BSA/0.1% NaN_3). An immunoconjugate was added (5 μg/ml final concentration), and the cells were incubated for 30 min either at 37°C for the *mfVIIasm* immunoconjugate or on ice for the *G71–1* immunoconjugate; the control cells were incubated without added immunoconjugate. After incubation the cells were washed with TBS/BSA, incubated 30 min on ice with fluorescein-labeled anti-human Fc γ-chain (Vector Laboratories), and analyzed on a Becton Dickinson FACsort instrument.

Adenoviral Vectors. The adenoviral vector system consists of the shuttle vectors pAdTrack-CMV and pShuttle-CMV and the backbone vector pAdEasy-1 (13). The immunoconjugate cDNAs were isolated from the pcDNA3.1 plasmid vectors by digestion with *Hin*dIII followed by Klenow fragment to fill in the 3′ recessed end, and then they were digested with *Not*I to release the cDNA insert, which was purified by agarose gel electrophoresis. The shuttle vectors first were digested with *Kpn*I followed by Klenow fragment, and then were digested with *Not*I. 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. Transformed colonies were selected on 2×TY/kanamycin agar, and the shuttle vectors were extracted and purified. The purified shuttle vectors and pAdTrack-CMV DNAs were digested with *Pme*1 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 1×10^5 293 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 10^{13} virus particles as estimated from the absorbance at 260 nm; the conversion is 1 OD unit = 1×10^{12} 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 5×10^5 TF2 or LXSN human melanoma cells. After the tumors had grown to a palpable size below the skin surface ($\approx$5 mm^3) or to a larger size above the skin surface ($\approx$50 mm^3), 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 $(\text{width})^2\,(\text{length})/2$. 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.

Immunohistochemistry. Paraffin sections of the tumors and organs were incubated in PBS + 0.3% H_2O_2 for 30 min and blocked in TBS/BSA buffer for 30 min. A solution containing 10 μg/ml the *mfVIIasm* immunoconjugate in TBS/BSA/Ca^{2+} 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

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 TF2 cells. The *mfVIIasm* immunoconjugate also was tested by immunohistochemistry for binding to sections of a human melanoma xenograft generated from the melanoma line LXSN/VEGF, which produces a high level of VEGF, resulting in a densely vascularized xenograft. Binding occurred to the tumor vascular endothelial cells as well as to the tumor cells (Fig. 3), indicating that TF is expressed by both cell types in the xenograft. Immunohistochemistry tests with sections of normal mouse liver, kidney, lung, and brain showed that the *mfVIIasm* immunoconjugate does not bind to vascular endothelial cells in these tissues, consistent with other evidence that TF is not expressed by vascular endothelial cells of nontumorous tissues (6, 7).

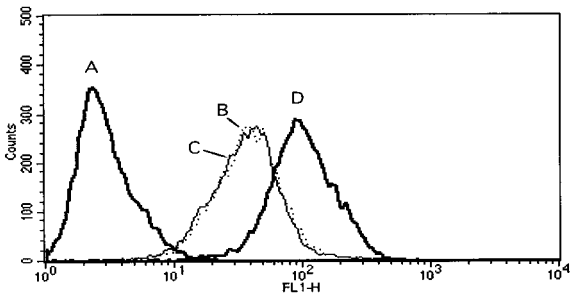

FIG. 1. Competition between human fVIIa and the *mfVIIasm* immunoconjugate for binding to TF2 cells. The assays were done by fluorescence-activated cell sorting. Curve A: Control without fVIIa or *mfVIIasm* immunoconjugate. Curve B: Equimolar mixture of fVIIa and *mfVIIasm* immunoconjugate (25 nM each). Curve C: 10× molar excess of fVIIa to *mfVIIasm* immunoconjugate (250 nM/25 nM). Curve D: *mfVIIasm* immunoconjugate only (25 nM).

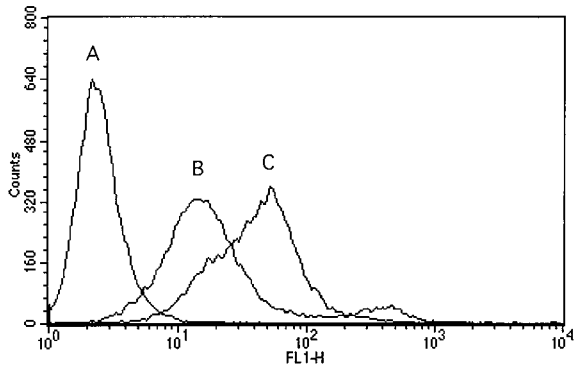

FIG. 2. Fluorescence-activated cell sorting assays for binding of the *mfVIIasm* immunoconjugate to LXSN and TF2 cells. Curve A: TF2 cells without *mfvIIasm*; curve B: LXSN cells with *mfvIIasm*; curve C: TF2 cells with *mfvIIasm*.

Immunotherapy Tests. 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 mm^3 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 mm^3,

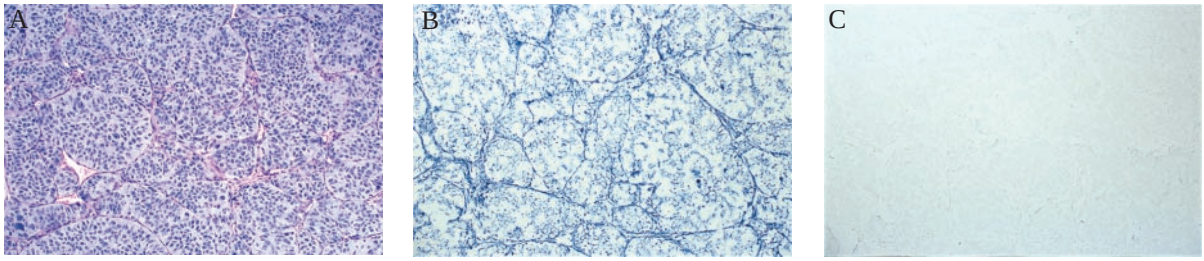

FIG. 3. Immunohistochemical assay for binding of the *mfVIIasm* immunoconjugate to tumor cells and tumor vascular endothelial cells in an LXSN/VEGF xenograft grown in SCID mice. The second antibody was anti-human γ-chain labeled with alkaline phosphatase, and the substrate was 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, which produces a blue color; the counterstain was methyl green. (*A*) Control stained with hematoxylin + eosin showing extensive vascularization of the xenograft. (*B*) Immunohistochemistry with the *mfVIIasm* immunoconjugate showing intense staining of both the vasculature and tumor cells. (*C*) Immunohistochemical control without the *mfVIIasm* immunoconjugate. Magnification: ×85.

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 mm^3 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 *fVIIasm* 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 mm^3 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 mm^3, 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

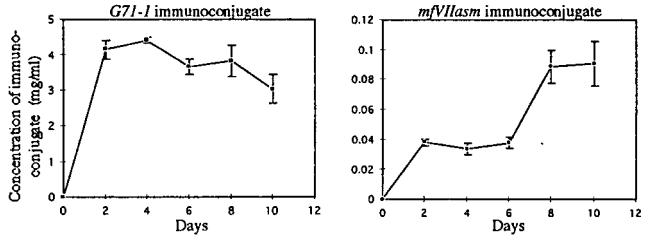

FIG. 4. Concentrations of the *G71–1* and *mfVIIasm* immunoconjugates in the blood of SCID mice after i.v. injections of the adenovirus encoding each immunoconjugate. The mice were injected on days 0 and 7 with 2×10^{11} adenovirus encoding the *G71–1* immunoconjugate or with 4×10^{11} adenovirus encoding the *mfVIIasm* immunoconjugate. The concentration of the encoded immunoconjugate in the blood was determined by ELISA. Each point is the average of the concentration for the five mice in each group.

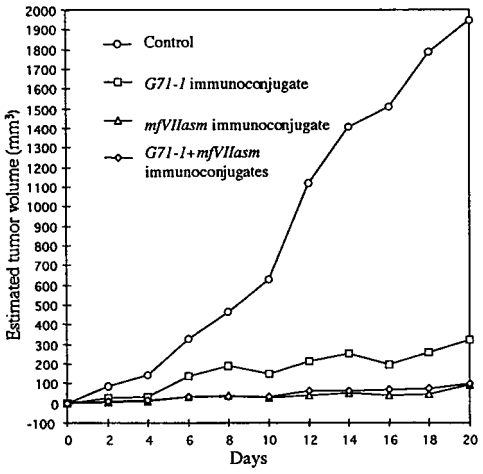

FIG. 5. Inhibitory effect of the *G71–1* and *mfVIIasm* immunoconjugates on the growth of a TF2 xenograft. For each curve five SCID mice were injected s.c. with 5×10^5 TF2 cells. When the xenografts had grown to a palpable size, the mice received tail vein injections on days 0, 7, and 14 of the adenoviruses indicated. The amount of adenovirus injected was 4×10^{11} for the control, 2×10^{11} for the adenovirus encoding the *G71–1* immunoconjugate, and 4×10^{11} for the adenovirus encoding the *mfVIIasm* immunoconjugate. The estimated tumor volumes are the averages for the five mice in each group.

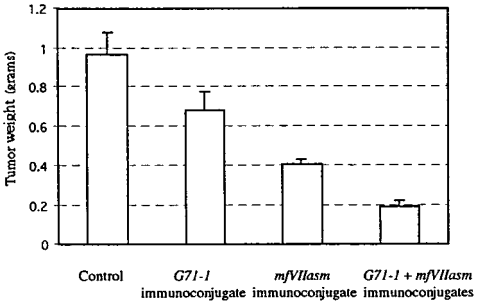

FIG. 6. Tumor weights of the xenografts from the experiment reported in Fig. 5. The xenografts were dissected from the mice on day 20, which was 6 days after the last injection of adenovirus. The bar heights are the average weights for the five mice in each group.

TF expressed in the brain vasculature or occurred spontaneously.

DISCUSSION

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 immunoconjugates, 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 Camelid heavy-chain antibody (19). For one type of immunoconjugate, 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 immunoconjugate, 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 fVII immunoconjugate to TF, an active site mutation was introduced into the mfVII targeting domain (*mfVIIasm)*, inhibiting the proteolytic activity required to initiate the blood coagulation pathway.

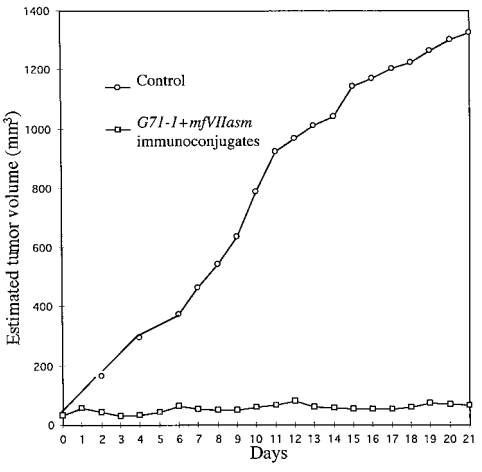

FIG. 7. Inhibitory effect of the *G71–1* and *mfVIIasm* immunoconjugates on the growth of a larger TF2 xenograft. Each mouse was injected s.c. with 5×10^5 TF2 cells, and the xenografts were allowed to grow to an estimated tumor volume of 50 mm^3 on the skin surface (day 1). A mixture of 2×10^{11} adenoviruses encoding the *G71–1* immunoconjugate and 7×10^{11} adenoviruses encoding the *mfVIIasm* immunoconjugate was injected into the tail vein of five mice on days 1, 6, 12, and 19. As a control five mice were injected with 4×10^{11} adenoviruses that did not encode an immunoconjugate. The estimated tumor volumes are the averages for the five mice in each group. One of the mice injected with the adenoviruses encoding the immunoconjugates was found dead on day 17; the estimated tumor volumes on subsequent days are the averages for the remaining four mice.

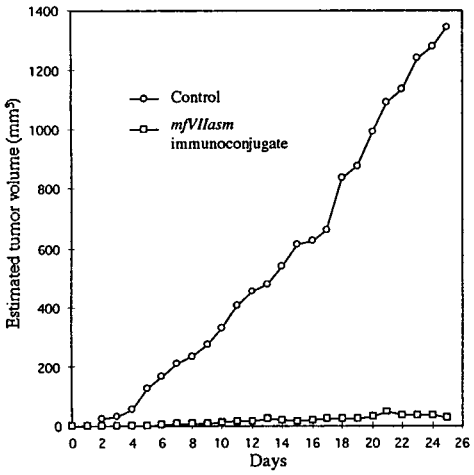

FIG. 8. Inhibitory effect of the *mfVIIasm* immunoconjugate on the growth of an LXSN xenograft. The mice were injected s.c. with 5×10^5 LXSN cells, and when the xenograft had grown to a palpable size (day 0) five mice were injected with 9×10^{11} adenoviruses encoding the *mfVIIasm* immunoconjugate, 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.

An earlier *in vitro* study showed that the *G71–1* immunoconjugate 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 immunoconjugates 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 immunoconjugates to SCID mice was achieved by tail vein injections of a replication-defective adenoviral vector encoding the immunoconjugates, 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 small ($\approx$5 mm^3) or larger ($\approx$50 mm^3) tumor before starting injections of the adenoviral vectors. Further growth of all the xenografts was prevented for the 3- to 4-week period of the experiments by multiple injections of the adenovirus encoding the *mfVIIasm* immunoconjugate, administered separately or together with the adenovirus encoding the *G71–1* immunoconjugate; in some of the mice the xenograft completely regressed. In the control mice, which were injected with an adenovirus that did not encode an immunoconjugate, the average volume of the xenografts increased by a factor of about 25 during the same period. In the mice receiving five injections of the adenoviral vectors encoding the immunoconjugates, many of the liver cells were enlarged and the spleen was infiltrated with erythrocytes. The defects were not caused by the secreted immunoconjugates, which do not bind to the liver or spleen cells. The primary cause probably is the continuous high-level synthesis of the encoded immunoconjugates by the liver cells, which are the mouse cells predominately infected by i.v. injected adenoviral vectors (18). The enlarged liver cells could have produced an increased blood pressure in the spleen, causing blood vessels to rupture. If these defects also can occur in a clinical setting, the problem might be corrected by changing the dose, schedule, or route of injection for the

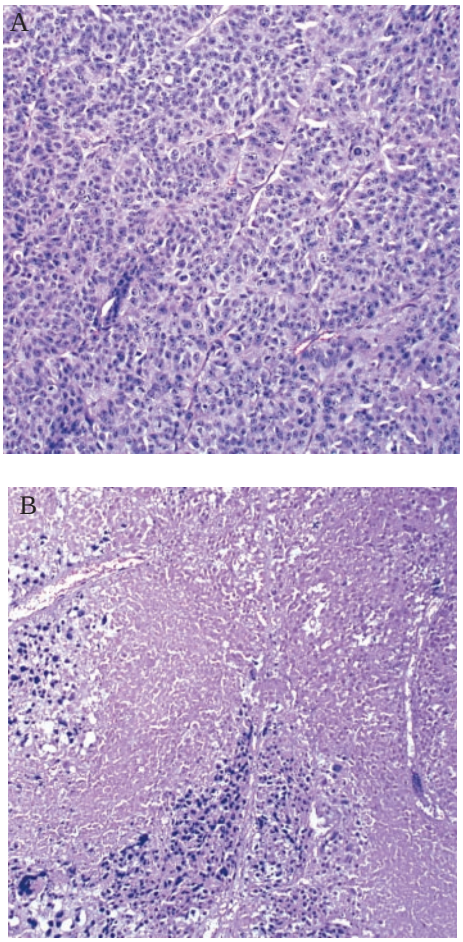

FIG. 9. Histochemistry of the LXSN xenografts from the experiment reported in Fig. 8. The xenografts were dissected on day 25 and embedded in paraffin, and sections were stained with hemotoxylin + eosin. (*A*) Xenograft from a control mouse injected with the adenovirus that does not encode an immunoconjugate.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

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 a fVII 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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