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Triggering of Antitumor Activity Through Melanoma-specific Transduction of a Constitutively Active Tumor Necrosis Factor (TNF) R1 Chimeric Receptor in the Absence of TNF-α[1]

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ABSTRACT

Tumor necrosis factor-α (TNF-α) has been intensively studied because of the specific toxicity of this cytokine toward cells that undergo malignant transformation. However, its proinflammatory and immunoregulatory properties always represented a drawback to the TNF-α administration in cancer therapy. In this study, we describe an adenovirus-based strategy in which the tumoricidal activity of TNF-α can be selectively triggered to eradicate tumors without administering TNF-α. This strategy might allow us to prevent TNF-α effects on normal tissues and, therefore, to bypass its systemic toxic effects. We inserted the constitutively active version of the M_r 55,000 TNF receptor, which was shown previously (F. Bazzoni *et al.*, Proc. Natl. Acad. Sci. USA, *92:* 5376–5380, 1995) to be capable of killing cells upon expression in the absence of its ligand, into a replication-deficient adenovirus, and under the control of a melanoma-specific promoter/enhancer element. We show that, upon infection, the recombinant gene reaches high level of expression in melanoma cell lines and triggers apoptosis by activating the caspase cascade. Expression and function of this receptor is restricted to melanoma cell lines, because morphology, viability, and proliferation of other cell types exposed to the recombinant adenovirus infection are not affected. We show for the first time that a TNF-like apoptotic response can be triggered in the absence of TNF-α and can be selectively confined to specific cellular targets. Killing activity and tissue specificity of the recombinant TNF receptor adenovirus, together with the advantage of triggering a TNF-like antitumor activity in the absence of TNF-α itself, are ideal features for a vector that might be the choice for gene therapy aimed to eradicate malignant cells.

INTRODUCTION

TNF-α[3] is a macrophage/monocyte-derived cytokine originally shown to be able to mediate tumor regression in experimental tumor models (1, 2). The attempts to make use of the tumoricidal property of this cytokine led to the development of several strategies, which, in some cases, resulted into the use of TNF-α in clinical trials of tumor chemoimmunotherapy (3).

TNF-α exerts its cytotoxic activity by triggering cellular apoptosis through the activation of two high-affinity receptors: the M_r 55,000 type I (TNFR1) and the M_r 75,000 type II TNF receptor (TNFR2; Ref. 4). TNFR1 has been regarded as the main cytotoxic signals transducer, the signaling pathway of which has been characterized in detail, whereas TNFR2, not yet devoid of cytotoxic activity, mainly induces cell proliferation, potentiates the role of TNFR1, and induces nuclear factor κB activation through a completely different signal transduction pathway (5). Both receptors are expressed on all of the nucleated cells; the fate of the cell exposed to TNF-α depends upon the levels of expression of either receptor, of the cytoplasmic transducing proteins, and of the nuclear factor κB-inducible cytoprotecting factors (6–8).

Systemic administration to cancer patients of TNF-α at high doses, required to obtain a curative response, resulted in severe toxicity (9) because of the proinflammatory and immunoregulatory properties of this cytokine and especially of its crucial role in the establishment of severe pathologies, such as cachexia and endotoxic shock (10). Several attempts have been made with the purpose of triggering only the TNF-α-specific tumor necrotic activity, bypassing its systemic toxic effects. With the aim of selectively inducing the TNF-α tumoricidal effect, overcoming at the same time the need to administer this cytokine, we sought to express a constitutively active version of the TNFR1 in malignant cells under the influence of a tissue-specific promoter. In a previous work, we have developed a constitutively active version of the TNFR1, which was shown to cause cell death in the absence of the extracellular ligand (11). This molecule consists of the extracellular portion of the Epo receptor joined to the stem, transmembrane, and cytosolic domains of the TNFR1 (Epo/TNFR1).

In this study, we describe the generation of a replication-deficient adenovirus able to transduce the constitutively active Epo/TNFR1, under the control of the melanoma-specific tyrosinase promoter/enhancer elements. We show that, upon infection, the recombinant gene reaches an efficient level of expression in the target cell population, whereas it is barely detected in other cell types. The results obtained in our experiments demonstrate for the first time that we can trigger a TNF-like apoptotic response in the absence of this cytokine through adenovirus-mediated overexpression of the constitutively active form of the TNFR1. Furthermore, by using a melanoma-specific promoter, which is not transcriptionally active in cells of different lineage, we show that the apoptotic response can be confined to melanoma cell lines that express tyrosinase.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The following cell lines were used in this study: H5V (mouse cardiac endothelial cells); SKmel28 and Skmel25 (human melanoma); HeLa (human cervix epithelioid carcinoma); and NIH 3T3 (mouse embryo). These cell lines were obtained from American Type Culture Collection (Rockville, MD), whereas fibroblast-like primary cell lines Hel 299 (human embryonic lung) and Hep 3B.1-7 (human hepatocellular carcinoma) were purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). The human melanoma cell lines 501mel (12) and a metastatic melanoma-derived cell line Me 2658 have been generated at the Cancer Immunotherapy Unit, Istituto Nazionale Tumori, Milan, Italy and kindly provided by Dr. Licia Rivoltini, together with B16F10 (mouse melanoma) and A375 (human melanoma). The cell line HT-29 (human adenocarcinoma) was a kind gift of Dr. M. Colombatti (University of Verona, Verona, Italy), and the cell line CFPAC1 (human pancreatic adenocarcinoma) was kindly provided by Prof. Nicholas R. Lemoine (Imperial Cancer Research Fund, London, United Kingdom). Adenoviruses were propagated on 293 (human embryonic kidney; adenovirus-5 transformed) parental cell line (pur-

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[3] The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; Ad, adenovirus; Epo, erythropoietin; CMV, cytomegalovirus; hGH, human growth hormone; FBS, fetal bovine serum; β-gal, β-galactosidase; MOI, multiplicity of infection; pfu, plaque forming unit; MTT, 3-(4,5-dimethylthiazol-2-year1)2,5-diphenyltetrazolium bromide; AMC, 7-amino-4-methilcoumarin; DEVD, Asp-Glu-Val; Ac, *N*-acetyl.

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chased from Microbix Biosystems, Ontario, Canada). All of the cell lines were maintained at 37°C in a 5% CO_2-humidified incubator and grown in DMEM (Bio Whittaker, Bergamo, Italy), supplemented with 100 units/ml penicillin/streptomycin and 10% FBS (Biochrom, Berlin, Germany).

Generation of Recombinant Adenoviruses. A basic plasmid pACPL, suitable for generation of recombinant adenoviruses, was generated by replacing the *Not*I fragment, which contains the CMV early promoter, the pUC19 polylinker, and the SV40 splice and polyadenylate signal sequences from pACCMV plasmid (obtained from Robert Gerard, University of Texas Southwestern Medical Center, Dallas, TX; Ref. 13), with a new polylinker (*Not*I, *Eco*RI, *Kpn*I, *Bam*HI, *Xba*I, *Sal*I, *Hin*dIII). A new construct named pACTyr-Epo/TNFR1 was engineered by the subsequent cloning into pACPL of: (*a*) 470 bp of the murine tyrosinase promoter/enhancer sequence, PCR-amplified from phsTyr(0.2)PCAT construct, kindly provided by Dr. R. Ganss (14), and cloned (*Eco*RI→*Kpn*I) into pACPL vector. This promoter consists of 270 bp of the minimum mouse tyrosinase promoter (15) and 200 bp *Ssp*I-*Xho*I fragment located at −12.3 to −12.1 kb of the 5′-flanking region of the mouse *tyrosinase* gene. This sequence has been described previously as the minimum element that provides melanoma-specific transcriptional enhancing activity (14); (*b*) the chimeric receptor Epo/TNFR1 cDNA (11), subcloned (*Kpn*I→*Sal*I) from pCMV5 vector into pACPL, downstream of the tyrosinase promoter/enhancer sequence; and (*c*) the hGH transcription termination and polyadenylate signals sequences, PCR-amplified from pCMV4 vector and cloned (*Sal*I→HindIII) at the 3′ end of the chimeric receptor cDNA.

A second recombinant plasmid for adenovirus generation, pACTyr-βgal, was produced. The *lacZ* gene product β-gal cDNA was cloned (*Xba*I→*Sal*I) in place of the Epo/TNFR1 cDNA. Both the β-gal and the Epo/TNFR1 cDNA expression were under the control of the same tyrosinase promoter/enhancer elements and hGH polyadenylation signal. The new constructs were sequenced and proven to be correct.

The Ad-Tyr-Epo/TNFR1 and Ad-Tyr-β-gal vectors were generated by *in vivo* recombination between pJM17 plasmid (Microbix Biosystems), which contains the adenoviral genome with a deletion of the E1 early genes region (16), and the shuttle plasmids pACTyr-Epo/TNFR1 and pACTyr-β-gal, in which the tyrosinase promoter and recombinant genes had been cloned. Primary plaques were isolated, amplified, and screened by Southern blot to verify the presence of the tyrosinase promoter/enhancer plus Epo/TNFR1 or β-gal cassette. Large-scale preparation of viruses was conducted as described previously (17). Viral titer was determined by plaque assay on 293 cells.

A third recombinant adenovirus, encoding the β-gal driven by the CMV promoter, was a kind gift of Dr. B. Beutler (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX) and was used to determine the optimal MOI at which each cell line would receive at least one copy of the recombinant gene. All of the three recombinant adenoviruses are schematically represented in Fig. 1.

Infection of Cell Lines and Analysis of β-gal Expression. All of the different cell lines were seeded in 96-well tissue culture plates at 10^4 cells/well. The day after, media were aspirated from each well, and the optimum amount of viral pfu was diluted in infection media (DMEM + 2% FBS) and added to the monolayers of the cells. The cells were incubated for 30 min at room temperature. Subsequently, complete medium was added, and the cells were incubated at 37°C, 5% CO_2 for the indicated lengths of time. Ad-Tyr-β-gal- and Ad-CMV-β-gal-infected cells were either fixed for *in situ* β-gal staining or harvested, and extracts were prepared for quantitative β-gal assays. *In situ* β-gal staining and quantification of β-gal enzyme activity were performed as described previously (18, 19). In the β-gal assay, fluorescence was measured on a microtiter fluorescence plate reader (Fluorocount; Packard; λ_{ex}, 350 nm and λ_{em}, 460 nm).

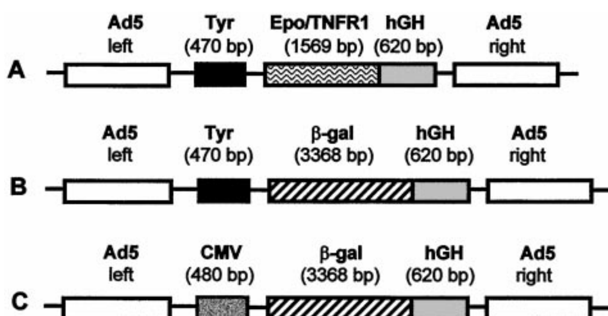

Fig. 1. Schematic representation of the recombinant Ad vectors, containing the Epo/TNFR1 cassette ▨ (*A*) or the β-gal cassette ▨ (*B* and *C*). *A* and *B*, the recombinant cDNAs are under the control of the Tyrosinase promoter/enhancer sequences ■. *C*, the β-gal cDNA expression is driven by the CMV promoter ▩. All of the recombinant adenoviral vectors use the hGH polyadenylation signal ▩. All of the vectors are E1-deleted Ad-5-based vectors.

Northern Blot Analysis. Cytoplasmic RNA was harvested from Skmel28 cells that were left untreated or infected with either Ad-Tyr-β-gal or Ad-Tyr-Epo/TNFR1 at the indicated time after infection, using an NP40 lysis technique as described previously (20), and was subjected to electrophoresis in an agarose gel. Northern blot analysis was performed on 20 μg of RNA/lane. Nylon blot was hybridized with the cDNA probe encoding the extracellular portion of the Epo receptor, labeled by the Ready to go kit (Pharmacia, Uppsala, Sweden), according to the manufacturer's instruction. The membrane was stripped and reprobed with the labeled β-gal-encoding cDNA. The extent of hybridization was quantitatively analyzed in the InstantImager (Packard Instruments, Meriden, CT).

Ligand Binding Assay. SKmel28 cells were plated at 3×10^5 cells/well in a 12-well plate, left to adhere, and infected with the recombinant adenoviruses Ad-Tyr-Epo/TNFR1 or Ad-Tyr-β-gal. At different times after infection, medium was replaced with 1 ml of DMEM, supplemented with 2% FBS, 25 mM HEPES, and 85 nCi of ^{125}I-labeled Epo (Pharmacia; specific activity of 300–600 Ci/mmol), in the presence or absence of 13 nM cold Epo (Eritrogen 100; Boehringer Mannheim, Milan, Italy). Cells were incubated for 4 h at 4°C with gentle shaking, harvested, and washed twice with cold PBS. Bound ^{125}I-labeled Epo was quantified with a gamma counter (1261 Multigamma counter; LKB Wollach).

Cytotoxicity Assays. SKmel28 cells were seeded in 96-well microtiter plate at 10^4 cells/100 μl/well. After an overnight incubation, cell monolayers were infected with 10^3 MOI of the indicated recombinant adenoviruses. Cell viability was assayed 24, 48, or 72 h after infection, either by crystal violet or MTT assays, as described previously (21, 22). Cell viability was reflected by dye absorbance, determined by absorbance measurements at 595 nm for crystal violet staining and at 540 nm for MTT assay, on an automated microplate reader (Argus 300; Packard).

Relative cell viability, in both assays, was calculated as follows:

$$\% \text{ viability} = \frac{\text{Experimental absorbance} - \text{background absorbance}}{\text{Absorbance of untreated controls} - \text{background absorbance}} \times 100$$

Fluorimetric Analysis of Caspase-3 Activity. SKmel28 cells (3×10^5/well) were seeded in a 12-well plate and infected with 10^3 MOI of Ad-Tyr-β-gal or Ad-Tyr-Epo/TNFR1 adenoviruses. At different time points after infections, medium was removed, and cells were resuspended in 160-μl lysis buffer [10 mM Tris-HCl, 10 mM NaH_2PO_4/$NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM Na PP_i] and incubated for 2 h at 37°C with the caspase-3 fluorogenic substrate Ac-DEVD-AMC (20 μM final; PharMingen, San Diego, CA) in 1-ml protease assay buffer [20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT]. Caspase-3 activity was proportional to the release of AMC from Ac-DEVD-AMC, measured using a spectrofluorometer (LS 50; Perkin-Elmer) with excitation and emission wavelengths of 380 nm and 440 nm, respectively. The assay was performed in the presence or absence of the broad spectrum caspase inhibitor DEVD-CHO (100 nM; PharMingen).

Protein concentration was determined using the Bio-Rad protein assay.

RESULTS

Cell Type Specificity of the Tyrosinase Promoter/Enhancer-containing Recombinant Adenoviruses. To test the transcriptional efficiency and lineage specificity of the tyrosinase promoter/enhancer elements, β-gal expression was evaluated in a panel of different human and murine cell lines infected with the Ad Tyr-β-gal virus, and data were compared with parallel infections with the Ad-CMV-β-gal virus. Both viruses were used at a 10^3 MOI, which represented the

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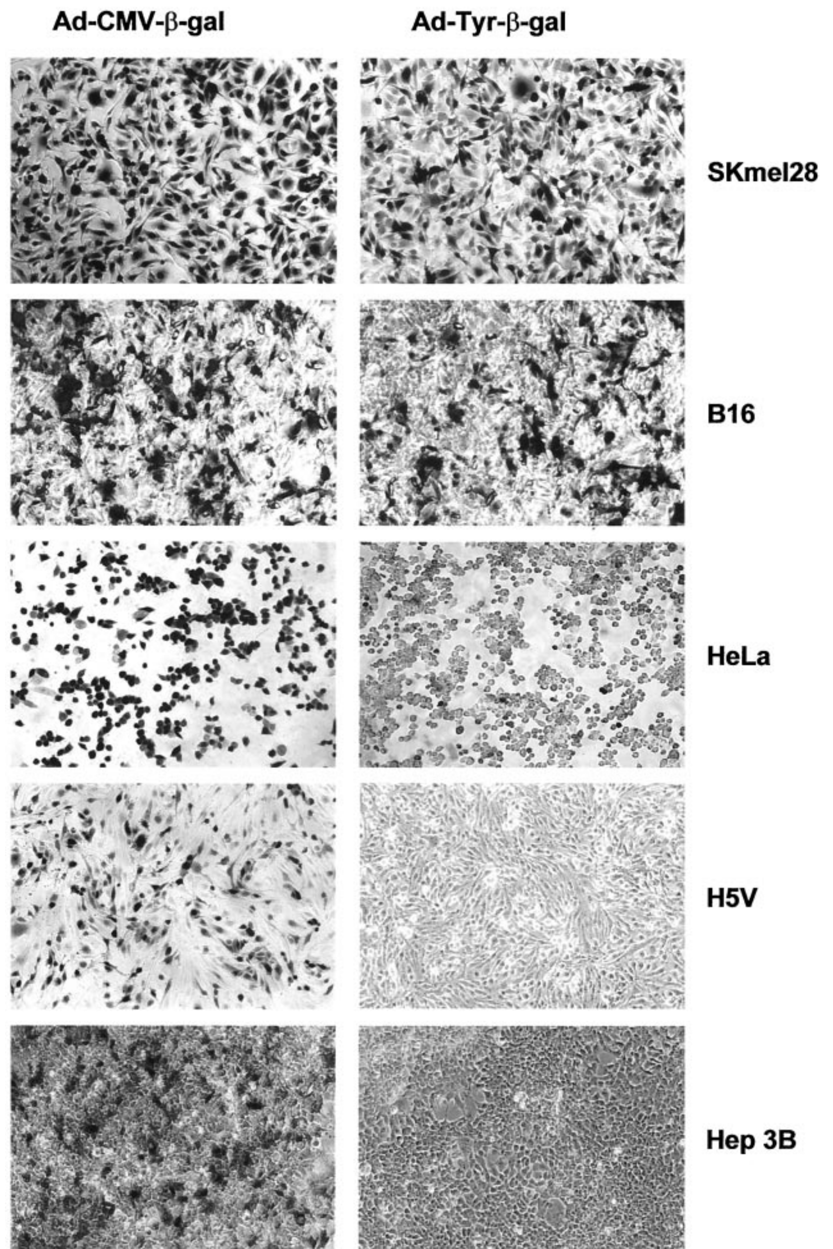

Fig. 2. Transcriptional efficiency of Tyr promoter *versus* CMV promoter. *In situ* staining for β-gal activity 24 h after infection of human Skmel28 and mouse B16F10 melanoma cell line, and HeLa, H5V, and Hep 3B nonmelanoma cell lines with Ad-CMV-β-gal (*left panels*) or Ad-Tyr-β-gal (*right panels*), performed as described in "Materials and Methods." Photomicrographs were taken at ×100.

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MOI at which a minimum of 50% of all of the cell lines in culture were infected. β-gal expression was evaluated 48 h after infection by both *in situ* β-gal staining (Fig. 2) and quantitative β-gal enzymatic assay (Table 1). Either the murine tyrosinase promoter/enhancer cassette or the CMV promoter, which is known to be a strong and widely used promoter in eukaryotic cells, drove high levels of β-gal expression in human melanoma SKmel28 and mouse melanoma B16F10 cell lines (Fig. 2), which are both known to express the tyrosinase gene. Nearly 100% of Skmel28 cells stained positive 48 h after infection with either adenovirus. The lower efficiency of infection observed in the B16F10 cells, compared with Skmel28, reflects the tropism of adenovirus, which is for human cells. Nevertheless, the percentage of B16F10 cells that express the β-gal after infection with Ad-CMV-β-gal or Ad Tyr-β-gal virus is comparable. Infection of these two melanoma cell lines (Skmel28 and B16F10) with the Ad-Tyr-β-gal resulted in high levels of β-gal enzymatic activity, which are 42 and 33%, respectively, of the activity of the CMV promoter (Table 1). On the contrary, in all of the other cell lines tested (HeLa, HT-29, and Hep 3B in Fig. 2; and H5V, NIH 3T3, CFPAC1, and Hel 299 in Table 1) and infected with the Ad-Tyr-β-gal, few blue-stained cells were visible in the *in situ* staining, whereas the β-gal activity evaluated in the enzymatic assay was negligible compared with that of cells infected with Ad-CMV-β-gal. In particular, β-gal activity induced by the tyrosinase promoter corresponded to 1.4% of β-gal activity induced by the CMV promoter in Skmel25, a human melanoma cell line in which no detectable levels of tyrosinase mRNA were found (23), whereas infection of other nonmelanoma cell lines, HeLa, H5V, and Hep 3B (Fig. 2; Table 1), and HT-29, NIH-3T3, CF PAC1, and Hel 299 (Table 1) with Ad-Tyr-β-gal resulted in very low absolute and percentage levels of β-gal expression, which ranged from 0.7 to 6% of that measured in Ad-CMV-β-gal infected cells. Adenoviral infection of these cell lines is not inefficient, because infection with the Ad-CMV-β-gal virus led to a high level of expression of the *β-gal* gene. Taken together, these data demonstrate the very high efficiency and specificity of the tyrosinase promoter/enhancer elements for melanotic melanoma.

Table 1 *Cell type specificity and transcriptional activity of the tyrosinase and CMV promoters*

Ad-Tyr-β-gal- or Ad-CMV-β-gal-infected cells were harvested at 48 h, and the levels of β-gal expression were quantified as described in "Materials and Methods." Data represent the mean ± SD of four independent experiments.

Cell lines	Ad-Tyr-β-gal	Ad-CMV-β-gal
Melanoma		
Skmel28	16647 ± 5305	39219 ± 7890
B16	13279 ± 2229	40288 ± 1463
Skmel25	325 ± 382	22343 ± 11881
Nonmelanoma		
HeLa	657 ± 181	44880 ± 6108
HT-29	1011 ± 303	21274 ± 2034
H5V	263 ± 14	32730 ± 1788
NIH-3T3	2364 ± 1331	38075 ± 547
CF PAC1	549 ± 33	25532 ± 1679
Hep 3B	276 ± 232	25890 ± 2450
Hel 299	317 ± 97	42184 ± 1523

To determine whether Ad-Tyr-Epo/TNFR1 was able to transduce the *Epo/TNFR1 chimeric receptor* gene, SKmel28 were infected with Ad-Tyr-Epo/TNFR1 at MOI of 10^3 pfu/cell. For comparison, cells were also infected with the adenovirus expressing β-gal under the control of the same melanoma-selective promoter. Expression of the two recombinant cDNAs was detected at different time points by Northern blot analysis (Fig. 3). Comparable levels of expression of Epo/TNFR1 receptor and β-gal were detected in transduced Skmel28 cells. β-gal mRNA was detectable 15 h after infection and continued to accumulate up to 64 h. Similarly, the tyrosinase promoter/enhancer elements were able to induce comparable levels of Epo/TNFR1 mRNA, which reached a plateau at 40 h and then decreased by 64 h, indicating that the exogenous Epo/TNFR1 cDNA was successfully transduced and efficiently and correctly transcribed into these cells.

Effect of Epo/TNFR1 Adenovirus-mediated Overexpression on Cell Growth and Viability. Adenovirus-mediated transduction of the Epo/TNFR1 molecule in SKmel28 and B16F10 cell lines resulted in an evident and massive cell death (Fig. 4*A*). SKmel28 and B16F10 cells infected with 10^3 MOI of the Ad-Tyr-β-gal showed a normal morphology and maintained a normal growth rate 72 h after infection, whereas cells infected with the same MOI of the Ad-Tyr-Epo/TNFR1 rapidly developed morphological characteristics typical of adherent cells undergoing apoptosis, which included membrane blebbing, rounded and shrunken shape, and detachment from the culture dish. Similar results were obtained when the same experiment was performed on other different melanoma cell lines. As shown in Fig. 4*A*, overexpression of the β-gal protein in 501mel and Me 2658 melanoma cell lines did not significantly alter their morphology and growth properties, whereas all of the cells expressing the *Epo/TNFR1* gene did rapidly undergo cell death. This effect was restricted to melanoma cell lines that were tyrosinase positive, because A375 amelanotic melanoma cell line was not affected by either virus infection (Fig. 4*B*, first column of panels). Similarly, cell lines of different lineage, such as H5V (endothelial), Hep 3B (hepatocellular carcinoma), and NIH 3T3 (mouse fibroblasts), in which the tyrosinase promoter was proven to be inactive in the β-gal assay (Table 1), were not sensitive to the Ad-Tyr-Epo/TNFR1 infection (Fig. 4*B*).

The strong and specific cytolytic effect induced by the Epo/TNFR1 expression is evidenced further by crystal violet staining (Fig. 5). SKmel28 cells were infected with Ad-CMV-β-gal, Ad-Tyr-β-gal, or Ad-Tyr-Epo/TNFR1 at a MOI of 10^3 pfu/cell. The heterologous gene expression was then evaluated by *in situ* β-gal staining only for the two viruses bearing the β-gal cDNA, whereas the cytolytic potential of each recombinant adenovirus was investigated by crystal violet staining (Fig. 5*A*). As indicated by the blue staining (Fig. 5*A*), 100% of the cells transduced with Ad-CMV-β-gal and Ad-Tyr-β-gal express high levels of β-gal 72 h after infection. However, intracellular accumulation of the β-gal protein did not affect the number of viable cells, and all of the cells were also stained positive for crystal violet. In contrast, Skmel28 cultures transduced with an identical MOI of the Ad-Tyr-Epo/TNFR1 were entirely destroyed; as a result, no crystal violet staining was observed. Quantitative evaluation of the Epo/TNFR1-induced cell death by MTT conversion and crystal violet-staining assay is shown (Fig. 5, *B* and *C*). The absorbance levels (at 540 nm for MTT and 595 nm for crystal violet) did not significantly differ between mock-infected and Ad-Tyr-β-gal-infected cells 48, 72, and up to 120 h (data not shown) after infection, which indicated that the events of adenovirus infection or β-gal overexpression did not impair cell viability. Epo/TNFR1 transduction of SKmel28 cultures resulted in greatly reduced absorbance levels both at 595 nm, which indicated a reduced number of intact cells in culture, and at 540 nm, which indicated the lack of chromogenic processing of MTT by the cells. Cell mortality was slightly but significantly appreciable (~33%) at 48 h, reaching 90% by 72 h. The cytolytic effect was comparable with that observed upon 24-h treatment with TNF-α plus cycloheximide (data not shown).

TNF-like Apoptotic Signal Triggered by the Ad-Tyr-Epo/TNFR1. Having established that Ad-Tyr-Epo/TNFR1 infection caused the death of cells that expressed the recombinant receptor, we investigated whether this death was the result of the intracellular accumulation of a toxic molecule *per se* or the consequence of a TNF-like apoptotic signal, triggered by the TNFR1 cytoplasmic domain. TNF-α, in fact, is known to activate a signaling pathway that leads to the activation of the caspase cascade through the TNFR1. Initially, we assessed whether the recombinant receptor correctly localized on the plasma membrane or simply accumulated in the cytoplasm after expression. Cells infected with the Ad-Tyr-Epo/TNFR1 were able to bind ^{125}I-labeled Epo in a specific manner because the addition of an excess of cold erythropoietin completely displaced the ^{125}I-labeled Epo bound to the receptor. No binding was detected in cells infected with the Ad-Tyr-β-gal (Fig. 6).

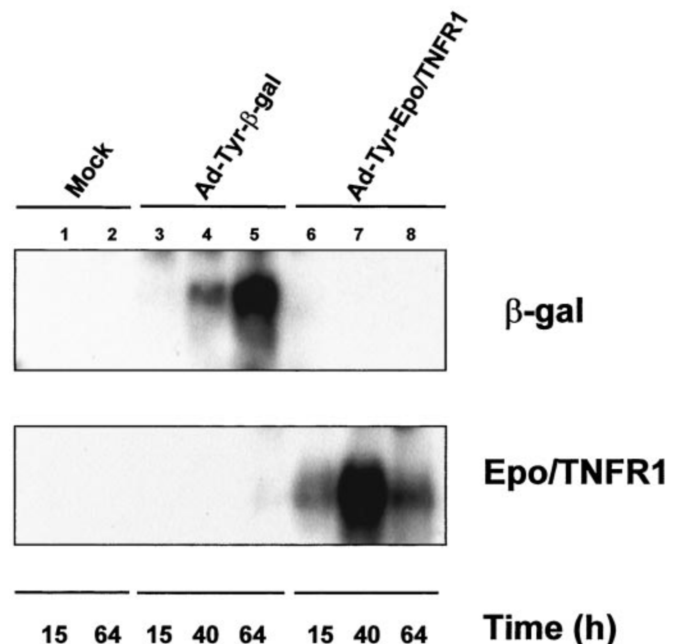

Fig. 3. Northern blot analysis. Time course expression of the Epo/TNFR1 and β-gal cDNAs transduced by the recombinant adenoviruses. Skmel28 cells have been mock infected (*Lanes 1* and *2*) and infected with Ad-Tyr-β-gal (*Lanes 3–5*) or with Ad-Tyr-Epo/TNFR1 (*Lanes 6–8*), both at 10^3 MOI. Cells were lysed, and cytoplasmic RNA was extracted at the times indicated after infection and analyzed by Northern blot with the β-gal and the extracellular portion of the Epo receptor cDNA probes, as indicated.

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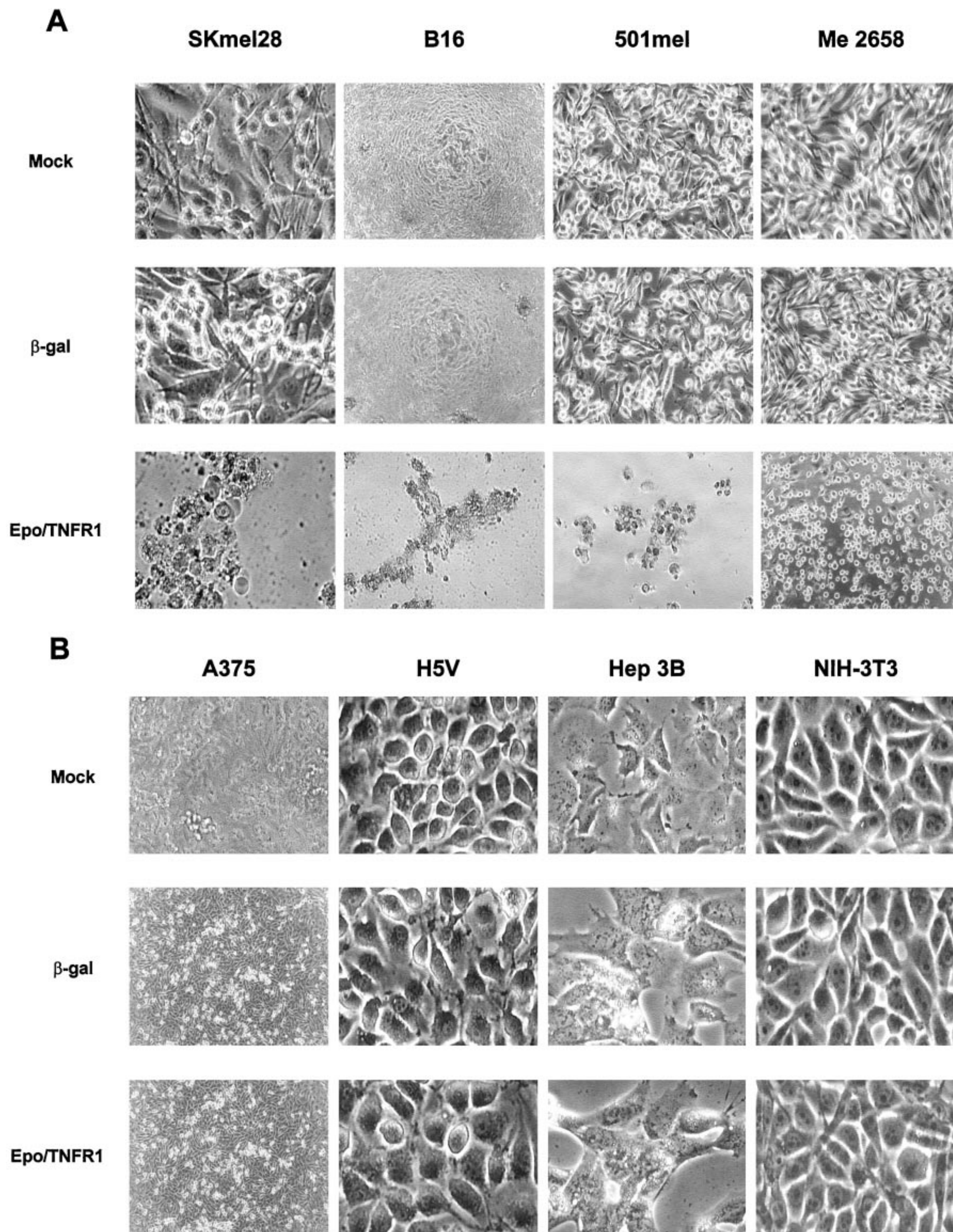

Fig. 4. Effects of Epo/TNFR1 and β-gal adenovirus-mediated overexpression. Photomicrographs were taken 72 h after infection of Skmel28, B16F10, 501mel, and Me 2658 (*A*); and of A375, H5V, Hep 3B, and NIH-3T3 (*B*) with 10^3 MOI of Ad-Tyr-β-gal or Ad-Tyr-Epo/TNFR1. *A*, all of the four melanoma cell lines, Skmel28, B16F10, 501mel, and Me 2658, infected with Ad-Tyr-Epo/TNFR1, appear altered in their morphology, shrunken, detached from the cell culture surface, and reduced in size. *B*, neither of the nonmelanoma cell lines, H5V, Hep3B, and NIH 3T3, nor the amelanotic melanoma cell line A375 show any morphological change after either recombinant adenovirus infection. Infection with the Ad-Tyr-β-gal did not impair the morphology of any cell type.

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Subsequently, measurement of caspase 3 activation revealed a marked increase in the levels of activated caspase in lysates of SKmel28 infected with the Ad-Tyr-Epo/TNFR1, whereas no significant activation of this caspase was observed either in cells transduced with the β-gal-encoding adenovirus or in the control mock-infected cells (Fig. 7). Caspase 3 activation was evident within 24 h after infection and continued to increase up to 48 h. By 72 h, caspase 3 activity of Ad-Tyr-Epo/TNFR1-infected Skmel28 was lower than that measured in mock-infected cells, which probably reflected the cell loss observed at this time point (data not shown). Treatment of SKmel28 cells with TNF-α plus cycloheximide resulted in a more rapid and transient activation of caspase 3, which reached the maximum levels within 24 h (data not shown). The caspase 3 activity was totally inhibited by the broad-spectrum caspase inhibitor DEVD-

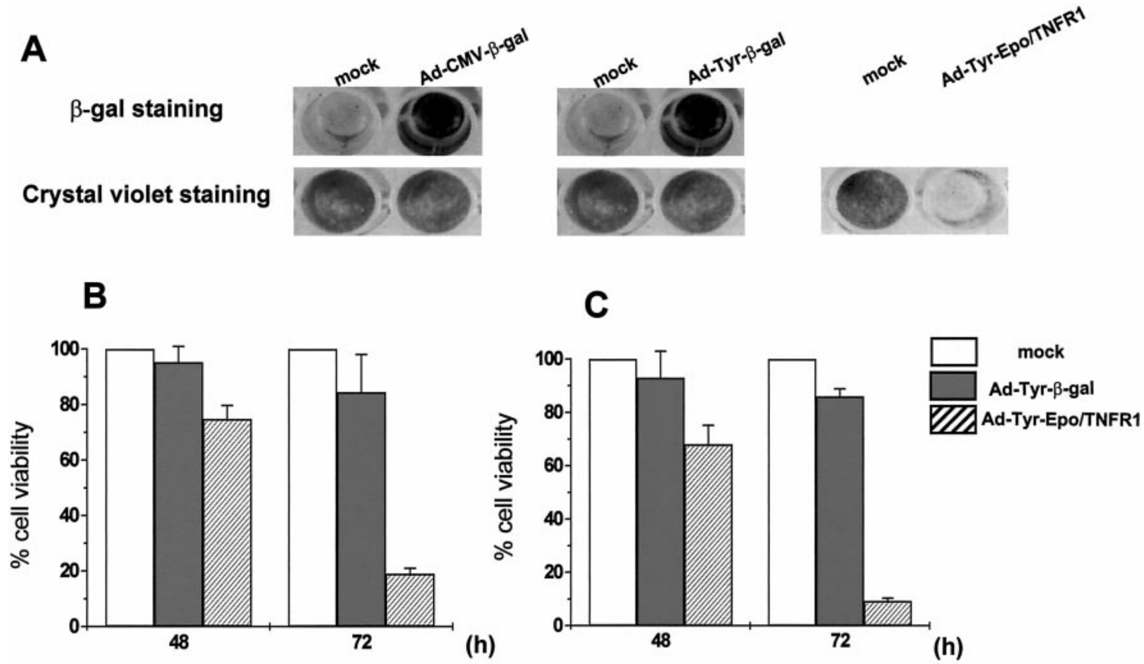

Fig. 5. Analysis of the Epo/TNFR1-induced cytotoxicity. *A*, 10^4 Skmel28 were mock infected (*first well*) or infected with 10^3 MOI of the indicated recombinant adenovirus (*second well*). The degree of heterologous gene expression was evaluated by *in situ* staining 72 h after infection for β-gal activity (*top wells*), and the degree of cell survival was evidenced by crystal violet staining (*bottom wells*). Quantification of cell death was performed by MTT assay (*B*) and crystal violet staining (*C*), as described in "Materials and Methods." Relative cell viability was calculated according to the formula described in "Materials and Methods." *B* and *C*, the mean values of four independent experiments ± SD.

CHO, indicating a causal role for caspase activation in the cell death process.

DISCUSSION

The antitumor activity of TNF-α is the earliest described feature of this cytokine, which has attracted scientific interest worldwide. However, soon after it became evident that studies on the antitumor activity of TNF-α *in vivo* were hampered by the need of systemically administering toxic doses of this cytokine to obtain a curative response. Unfortunately, the dark side of the TNF-α tumoricidal activity is represented by a wide variety of biological responses triggered by TNF-α through the activation of either the M_r 55,000 (TNFR1) or the M_r 75,000 (TNFR2) TNF receptor, expressed on all of the nucleated cells. All of the biological activities triggered by TNF-α in different physiological and pathological conditions point out the major role of this cytokine as a potent proinflammatory mediator and an important modulator of the immune system development and function, which is responsible for severe acute inflammatory reactions, of the establishment of cachexia in neoplastic patients, and of several autoimmune pathologies. Nevertheless, the attempts to exploit TNF-α-specific tumoricidal properties have not been dropped and have been directed either toward confining the TNF-α activity to the site of tumor by regional application of TNF-α through isolated limb perfusion (24–25) or toward constructing novel molecules that retain the full TNF-α antitumor activity while exhibiting reduced proinflammatory effects (26–28).

In this work, we developed a strategy aimed at triggering the cytolytic activity that TNF-α physiologically exhibits toward malignant cells while avoiding the administration of this cytokine. We made use of the Epo/TNFR1 chimeric receptor, which we have described previously as being able to trigger apoptosis upon overexpression in

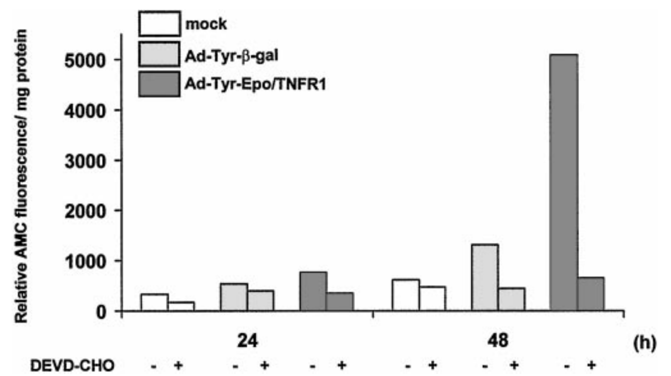

Fig. 7. Fluorometric analysis of caspase 3 activity. Caspase 3-dependent AMC formation was fluorometrically quantified at 24 and 48 h in mock-infected, Ad-Tyr-β-gal-infected, or Ad-Tyr-Epo/TNFR1-infected Skmel28 lysates, in the absence (−) or presence (+) of 100 nM of the caspase inhibitor DEVD-CHO. One experiment representative of three is shown.

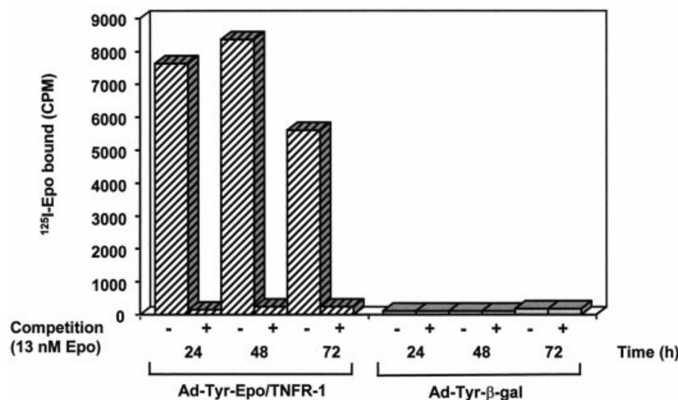

Fig. 6. Epo binding assay. Skmel28 cells were infected with Ad-Tyr-Epo/TNFR1 or Ad-Tyr-β-gal and subsequently incubated with 85 nCi ^{125}I-labeled Epo, in the presence (+) or absence (−) of 13 nM cold Epo, as described in "Materials and Methods." The amount of labeled Epo bound to the chimeric receptor was quantified in a gamma counter. One experiment representative of three is shown.

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the absence of the extracellular ligand, by virtue of the presence of the cytoplasmic domain of the TNFR1 and of the substitution of the endogenous TNFR extracellular domain with that of erythropoietin (11). We insured the efficiency of our system by the use of a recombinant replication-deficient adenovirus, which is known to be an efficient gene transduction system capable of infecting 100% of cells of epithelial origin in culture. Furthermore, direct injection of adenoviruses into certain tumors *in vivo* has been shown to lead to exceptionally high transduction efficiency near the site of injection (29). Therefore, the chimeric receptor, which is made of the extracellular domain of the erythropoietin receptor and of the stem, transmembrane, and cytoplasmic domains of the M_r 55,000 TNF receptor, was introduced into the E1 region of a replication-deficient adenovirus type 5.

Together with an efficient cell killing, the targeted expression to specific tissues or cells is a necessary feature for a gene vector therapy aimed at eradicating unwanted cells. We met these requirements by targeting the expression of the suicide gene to melanoma cells through cloning the recombinant Epo/TNFR1 cDNA under the control of the melanoma-specific tyrosinase promoter/enhancer sequence. Tyrosinase catalyzes the rate-limiting step in the synthesis of melanin, and it is expressed in pigmented cells of different developmental origin, including melanocytes, which are derived from the neural crest, and pigmented retinal epithelium, which is derived from the neural ectoderm (30–32). Indeed, other studies have shown the possibility of using the melanin synthetic pathway for *in vitro* and *in vivo* targeting of gene expression to melanoma cells (33–35).

In this study, we show that the melanoma-targeted expression of the chimeric Epo/TNFR1 receptor delivered by recombinant adenovirus is suitable for selective killing of melanoma cell lines by means of activation of a TNF-like apoptotic process. We initially demonstrated that the transcriptional activity of the tyrosinase promoter/enhancer elements, although lower than that of CMV promoter (42% in Skmel28 and 33% in B16F10), was still high enough to allow high levels of expression of the heterologous gene. By comparison with the CMV-promoted virus, the Tyr promoter was shown to be transcriptionally active in pigmented human (Skmel28) and mouse (B16F10) melanoma cell lines but highly regulated when tested in a panel of cells belonging to different lineage (HeLa, HT-29, NIH-3T3, CF PAC1, Hep 3B, and Hel 299) or to a tyrosinase-negative melanoma lineage (Skmel25). These data proved that the regulatory sequences derived from the mouse tyrosinase promoter were sufficient for high level, tissue-specific transcription in pigmented cells and indicated that this promoter would allow expression of the chimeric Epo/TNFR1 receptor in a regulated manner. Indeed, the expected cytolytic potential of the Epo/TNFR1 molecule (11) was observed upon infection of Skmel28 and B16F10 cells, in which the tyrosinase promoter/enhancer was proven to be active. Infection of these cell lines with the Ad-Tyr-Epo/TNFR1 virus triggered a series of events that ultimately led to the death of the transduced cells. Skmel28 and B16 undergo radical morphological changes, representative of the apoptotic process, 72 h after infection. Extension of this analysis to other pigmented melanoma cell lines (501mel and Me 2658) demonstrated that the cytolytic effect is not a consequence of a specific characteristic of Skmel28 and B16, but it is a general effect of Epo/TNFR1 expression in melanoma cell lines. Furthermore, the Epo/TNFR1 expression and consequent effects are confined to cells in which the tyrosinase promoter/enhancer elements have been proven to be active. In fact, cells of different lineage (H5V, Hep 3B, and NIH-3T3) that did not express the *β-gal* gene (Table 1) were not able to express the *Epo/TNFR1* gene either and, therefore, were not affected at all by the Ad-Tyr-Epo/TNFR1 infection (Fig. 4*B*). In the same manner, nonpigmented melanoma cell lines such as A375 (Fig. 4*B*) and Skmel25 (data not shown) were left unchanged by Ad-Tyr-Epo/TNFR1 infection, indicating that the expression of the proapoptotic molecule is not only confined to cells of the melanoma lineage but, among them, is restricted to cells in which the melanin metabolic pathway is active. Of critical importance are the low levels of expression observed in the endothelial cell lines (H5V), embryonic lung-derived primary cell lines (Hel 299), and hepatocellular carcinoma cell line (Hep 3B). Lack of expression at these sites will eliminate one source of potential toxicity, because endothelial cells together with pulmonary and hepatic tissues are preferential sites of viral infection after any route of viral administration. Overexpression of the recombinant receptor brings cells to death by triggering a series of events that departs from the plasma membrane and leads to the caspase cascade activation. Indeed, in Skmel28, the Epo/TNFR1 mRNA expression was detected as early as 15 h (Fig. 3), membrane localization of the receptor and functionality in binding the ligand were observed by 24 h (Fig. 6), and caspase 3 activation peaked at 48 h. All together, these events preceded the morphological changes, which were observed in the transduced cells between 48 and 72 h. By 72 h after infection, none the Skmel28 in culture survived. Parallel to the decrease in cell survival, Epo/TNFR1 mRNA, Epo binding, and caspase 3 activation declined by 72 h, indicating the progressive cell loss.

In several different gene therapy strategies, TNF-α represented the apoptosis-triggering molecule of choice. These strategies were based on the adenoviral-mediated transduction of tumor cells with TNF-α itself (36, 37), TNFR2-specific mutant of TNF-α (28), uncleavable membrane-bound mutant of TNF-α (38), or *TNFR1 receptor* gene alone (39) or in conjunction with TNFR1-specific TNF-α mutein administration (27). The direct intratumoral production of either a secreted or a nonsecreted form of TNF-α or of TNFR2-specific TNF-α mutant allowed high concentration of this cytokine to be reached locally and resulted in a relatively large reduction in systemic toxicity with little or no reduction in antitumor activity. However, it cannot be excluded that TNF-α that is produced as soluble product may exert effects (including toxic effects) at distant sites. This is, in fact, the main concern of the clinical application of TNF-α in cancer, which has been overcome through administration via isolated limb perfusion (3). This approach is based on the same rationale of physically confining TNF-α action to the tumor site. On the other hand, the strategy based on transduction of the TNFRs (26, 38) carries the same concerns of the previous one, because it requires the subsequent administration of TNF-α. Obviously, the safest way to avoid toxicity consists in not administering the toxic drug. Our results show, for the first time, that this goal can be achieved by the use of constitutively active receptors, such as those we have engineered, that allow us to overcome the TNF-α "bystander" effect of the systemic toxicity. We demonstrate that, in our system, safety is insured by the capability of avoiding TNF-α administration, which still mimes the TNF-α apoptotic action, and high selectivity is offered by the utilization of a melanoma-specific promoter.

The outcome of this study indicates that this strategy, which combines the cytolytic effect of the recombinant Epo/TNFR1, the lack of the need of administering TNF-α, together with the targeting to specific tissues, may represent a possible tool for the treatment of an aggressive cancer such as melanoma, the incidence of which is rapidly growing among the population.

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