

Adenovirus Infection Enhances Killing of Melanoma Cells by a Mitotoxin¹

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Abstract

Toxins are effective in cell killing if internalized efficiently. Conjugation of the plant toxin saporin with basic fibroblast growth factor has increased tumor killing due to better internalization, but toxin uptake by cells has remained relatively inefficient. We show here that infection of melanoma cells with a replication-defective adenovirus enhances cell killing by the mitotoxin basic fibroblast growth factor-saporin more than 10-fold, thus allowing tumor cell killing *in vivo* at nontoxic concentrations. Adenovirus infection leads to increased apoptosis by the mitotoxin due to enhanced internalization of the ligand-receptor complex and release of the active toxin from the endosomes.

Introduction

SAP³ is a plant mitotoxin that irreversibly inhibits protein synthesis in eukaryotic cells by inhibiting binding of the 60S ribosome subunit to elongation factor 2 (1). Conjugation to bFGF increases SAP killing of tumor cells, including melanoma (2–4). Exogenous bFGF is bound by FGF receptors 1 and 2, which are both expressed by melanoma cells (5, 6). Ligand-induced receptor-mediated endocytosis occurs through clathrin-coated vesicles. Receptors and ligands are sorted in acidified endosomes and then recycled to the cell surface, stored intracellularly, transported to the nucleus, or degraded in lysosomes (7, 8). Therefore, cytotoxicity of a mitotoxin depends upon efficient internalization, sorting, and release of intact mitotoxin into the cytoplasm. In the presence of endogenous bFGF produced by melanoma cells for autocrine growth stimulation, down-regulation of its receptors on tumor cells may lead to reduced internalization of the exogenous bFGF-SAP complex (9). bFGF-SAP cytotoxicity data also suggest a need for excess bFGF-SAP to induce killing, although one molecule of SAP may be sufficient to kill a single tumor cell (2). Thus the killing of cells by bFGF-SAP depends not only on internalization of the ligand-receptor complex, but more so on the release of the active compound from endosomes. It has been shown that adenoviruses possess a poorly understood mechanism that accomplishes cell vesicle escape (10, 11). In the present study, we show that bFGF-SAP acts synergistically with a replication-defective adenovirus to induce apoptosis, suggesting a potential application in cancer therapy. Through more efficient internalization and release, we could decrease effective treatment concentrations of SAP and reduce its toxicity in animals.

Materials and Methods

Materials. Recombinant bFGF-SAP and immunotoxin 11A8-SAP were a gift from Prizm Pharmaceuticals (San Diego, CA; Ref. 2). Ad5 containing the *LacZ* reporter gene was produced in human 293 cells; its purification and titration were done using standard protocols (12). Melanoma cell lines were established in the laboratory from primary and metastatic lesions (13), and adrenocorticocarcinoma cell line SW13 was obtained from American Type Culture Collection (Rockville, MD).

Cell Growth Assays. For [³H]thymidine incorporation assays, cells were seeded in 96-well plates at 3×10^4 cells/well for 24 h in MCDB153/L15 medium with 2% FCS. The cells in quadruplicates were treated with SAP, bFGF-SAP, bFGF, or Ad5 alone or in combination for 12 h. Forty-eight h later, the cells were pulsed with [³H]thymidine for 4 h before harvesting in an automatic cell harvester. The experiments were repeated three times. Anchorage-independent growth in soft agar was tested with cell treatments starting at the time of seeding. Colony-forming efficiency was determined in triplicates after 2 weeks. *In vivo* tumor growth was tested by s.c. injection of 1×10^6 1205Lu melanoma cells into the flanks of eight RAG-1 mice/group. Intratumoral injections were initiated after 10 days, followed by another injection 7 days later. Tumor growth and animal weight were measured biweekly for 45 days.

***In Vitro* Translation Assays.** Assays were done in a TNT-coupled reticulocyte lysate system using [³H]leucine and luciferase translation reactions (Promega, Madison, WI). The reaction mixtures in triplicate were treated with the mitotoxin or control preparations, initiated with T7 RNA polymerase, incubated for 60 min, precipitated on GF/C Whatman filter paper with 10% trichloroacetic acid, and counted after extensive washes.

Apoptosis Assays. Cell cycle distribution and apoptotic DNA profiles of melanoma cells were performed by staining cells with propidium iodide and measuring fluorescence in an EPIC XL flow cytometer (14). Quantitation of apoptosis by TUNEL was performed using FITC-labeled dUTP incorporation into DNA strand breaks (15). DNA fragmentation was analyzed on a 2% agarose gel.

Results

bFGF-SAP is 100-fold more cytotoxic for melanoma cells than SAP alone (2, 9) and reduces [³H]thymidine incorporation <10% at 1.0 nM (Fig. 1A). However, the efficiency of the conjugate at killing melanoma cells that express FGF receptors remains relatively low, and an estimated 20,000 molecules/cell are needed to kill melanoma cells, despite the fact that approximately 1 molecule/cell of SAP should be sufficient for killing (2). To decrease the required concentrations of bFGF-SAP by increasing the efficiency of internalization, we infected melanoma cells at the time of treatment with Ad5, an adenovirus containing the bacterial reporter gene *LacZ*. Infection of melanoma cells with 10 pfu/cell of Ad5 did not significantly decrease the [³H]thymidine incorporation after 48 h (Fig. 1A) and minimally reduced their soft agar growth (Fig. 1B). SAP alone or bFGF-SAP at 1 nM was also not effective in cell killing. On the other hand, combining bFGF-SAP treatment of cells with Ad5 infection reduced the growth of all five melanoma cell lines tested (WM164, WM115, WM852, 451Lu, and 1205Lu) and reduced the growth of the bFGF-responsive adrenocorticocarcinoma cell line SW13 in [³H]thymidine incorporation and soft agar growth assays by more than 85%. Thus,

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³ The abbreviations used are: SAP, saporin; Ad5, replication-defective adenovirus lacking E1 and E3 regions; bFGF, basic fibroblast growth factor; pfu, plaque-forming unit; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; FGF, fibroblast growth factor.

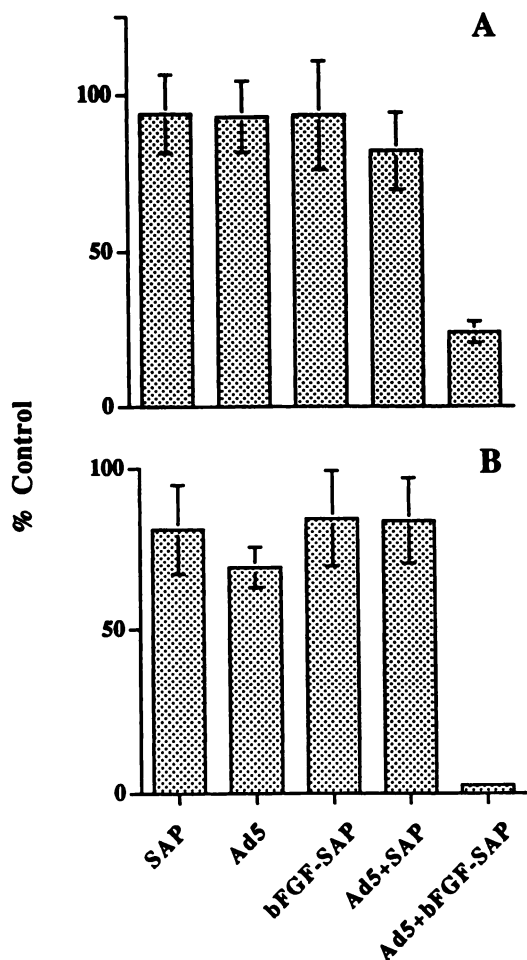


Fig. 1. Melanoma growth inhibition *in vitro* by the mitotoxin bFGF-SAP and replication-defective Ad5/LacZ. Melanoma cells were treated with 1 nM SAP or bFGF-SAP alone or infected with 10 pfu of Ad5/LacZ. A, inhibition of methyl-[³H]thymidine incorporation. B, inhibition of colony formation in soft agar. Results are expressed as a percentage of untreated control cultures from three experiments. Experiments were performed in quadruplicate (A) or triplicate (B).

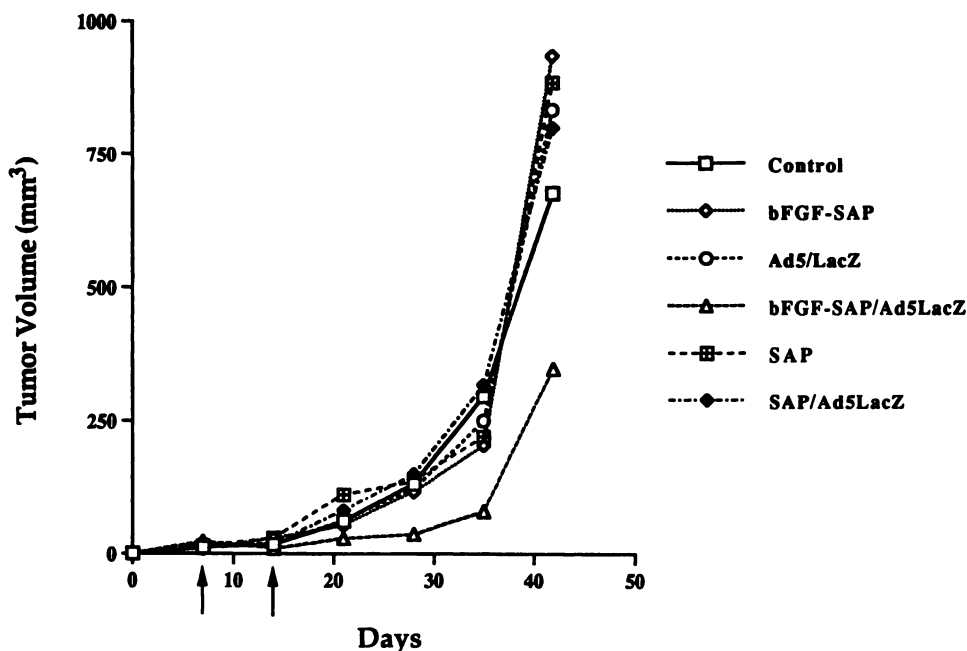
nontoxic concentrations of Ad5 and bFGF-SAP synergistically inhibit DNA synthesis and cell survival.

Melanoma tumor growth in RAG-1 mice was inhibited significantly by combining intratumoral treatment of bFGF-SAP with Ad5 infection (Fig. 2). SAP, bFGF-SAP, Ad5 with SAP, or Ad5 alone did not inhibit tumor growth significantly. bFGF-SAP plus adenovirus-treated animals showed no weight loss for the duration of the experiments and seemed healthy. Contrarily, effective doses of bFGF-SAP given without Ad5 caused 10–20% weight loss in most animals and over 20% mortality within 4 weeks (data not shown).

The ability of SAP and FGF-SAP alone or in combination with Ad5 to inhibit protein synthesis was determined using a rabbit reticulocyte lysate in a cell-free system that measures leucine incorporation into acid-precipitable material (Fig. 3). Nontoxic concentrations of SAP or bFGF-SAP were used to examine this effect. The toxins inhibited the protein synthesis by nearly 50%, and the addition of Ad5 did not significantly alter the ribosome-inactivating property of the toxins. These results show that Ad5 does not alter the mechanism of cytotoxicity of the toxins but are consistent with virus enhancement of uptake of bFGF-SAP.

Cells treated with Ad5/LacZ and bFGF-SAP showed microscopically typical apoptotic patterns of cell killing. These included initial morphological changes such as rounding, blebbing on the plasma membrane, and the presence of apoptotic bodies. Apoptotic cell death was measured in three assays: (a) TUNEL assay; (b) propidium iodide staining of the DNA; and (c) internucleosomal fragmentation of the DNA. The first evidence of apoptosis was seen after 24 h; in cells treated with bFGF-SAP and infected with Ad5, this increased to 50% by 72 h (Fig. 4A). Cells treated with SAP and bFGF-SAP alone or infected with Ad5 alone did not show significant numbers of apoptotic cells. About 15% apoptotic cells were seen after 72 h in cultures treated with SAP and infected with Ad5, suggesting that the virus infection can also increase the unconjugated toxin uptake, albeit at lower efficiency. FITC-conjugated propidium iodide incorporation analysis for apoptosis of treated melanoma cells confirmed the results of TUNEL assays (Fig. 4B). Only cells treated with bFGF-SAP and infected with Ad5 showed a significant fraction <2N, thus falling into the sub-G₁ fraction that is characteristic of apoptosis. Melanoma cells treated with bFGF-SAP and infected with Ad5 showed a ladder of

Fig. 2. Inhibition of 1205Lu melanoma growth in RAG-1 mice after treatment with bFGF-SAP and Ad5/LacZ. Melanoma cells were injected s.c.; 10 and 17 days later (arrows), tumor nodules were injected with 50 μ l of either SAP at 125 μ g/kg, bFGF-SAP at 200 μ g/kg plus Ad5/LacZ at 1×10^{-7} pfu, SAP plus Ad5/LacZ, Ad5/LacZ, or PBS. Tumor volumes were measured twice a week.



DNA fragmentation that coincided with the $<2N$ DNA peak by propidium iodide staining (data not shown), thus confirming that the combination treatment leads to apoptotic cell death.

Discussion

Bacterial or plant toxins such as diphtheria toxin, *Pseudomonas aeruginosa* exotoxin, ricin A chain, or SAP are potent cytotoxic agents that have been used in experimental and clinical cancer therapies. Because of their nonselectivity and poor permeability, they have been conjugated to antibodies and used as immunotoxins or to growth factors and used as mitotoxins. Chemically conjugated or genetically engineered bFGF-SAP shows more selective killing of tumor cells than SAP alone (2–4, 16). However, an excess of bFGF-SAP mitotoxin is still required for effective cell killing, limiting its usefulness due to nonspecific toxicity in animals. We show here that replication-defective adenovirus can significantly potentiate the cytotoxic activity of bFGF-SAP, at concentrations that are nontoxic *in vitro* and *in vivo*. This enhanced cytotoxic effect of bFGF-SAP is due to the ability of adenovirus to facilitate its release inside the cells. Once within the cytoplasm, SAP triggers apoptosis, similar to the bacterial toxin ricin, a ribosome-inactivating enzyme (17).

Adenoviruses enter cells by receptor-mediated endocytosis, and they can overcome degradation in the endosomes for release into the cytoplasm. Earlier studies by Pastan and coworkers (18) have demonstrated the utility of adenovirus to deliver protein-conjugated cytotoxins. The adenovirus enters the cells by the attachment of its fiber knobs onto an unidentified cell surface receptor. The viral penton base protein then associates with a fibronectin-binding integrin such as $\alpha\beta3$ or $\alpha\beta5$, thereby facilitating endocytosis of the virion through coated pits and vesicles (19). The extrusion of the virion from the endosome into the cytoplasm is mediated by acid-triggered lysis of the endosomal membrane, probably involving integrin(s), penton base protein, and a viral protease (20).

Adenoviruses alone are unable to significantly increase uptake of SAP, except after prolonged (72 h) incubation (Fig. 3A). Thus bFGF is required for internalization by binding to high-affinity FGF receptors 1 and 2, which are both expressed by melanoma cells (5, 6). The adenovirus apparently prevents degradation of the mitotoxin in endo-

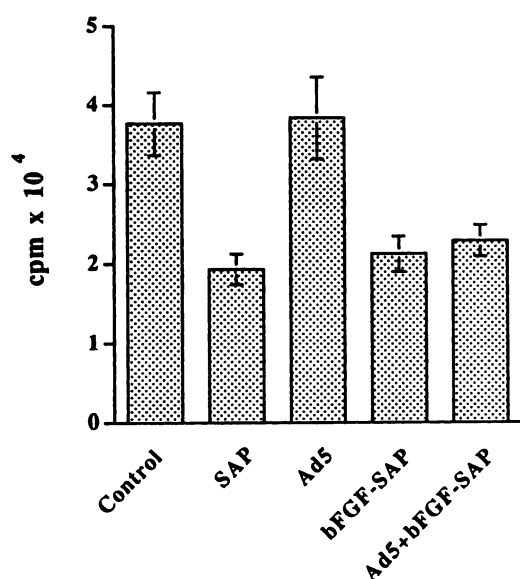


Fig. 3. Cell-free protein synthesis is inhibited by SAP but not Ad5/LacZ. *In vitro* translation was determined in a rabbit reticulocyte lysate system using [³H]leucine. Results show cpm incorporated and precipitated after polymerase initiation and luciferase translation. Results are average of two experiments performed in duplicate.

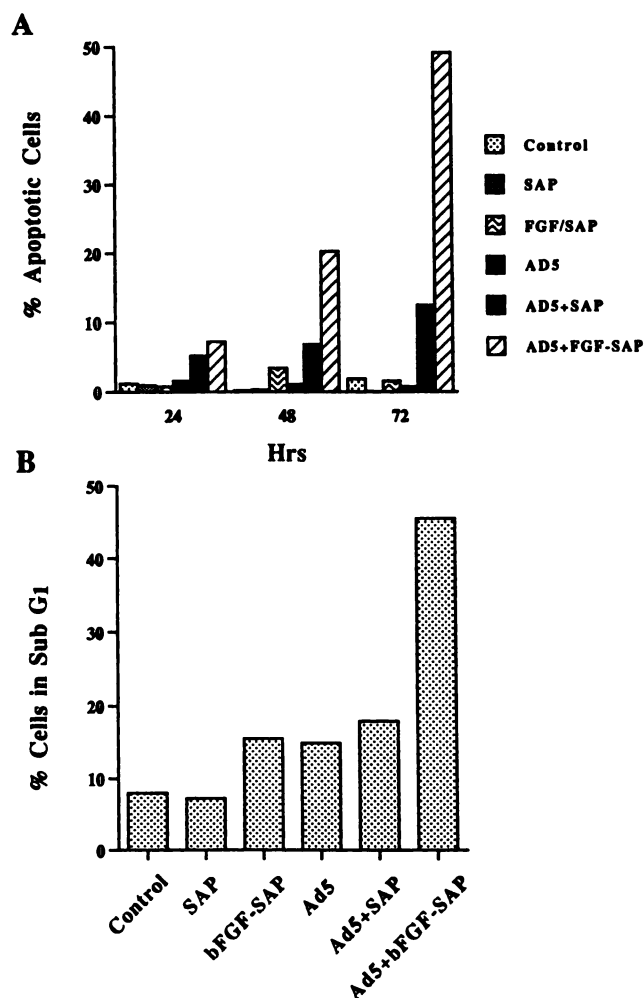


Fig. 4. Induction of apoptosis in melanoma cells by bFGF-SAP and Ad5/LacZ. WM164 melanoma cells were treated with SAP, FGF-SAP, and Ad5/LacZ alone or in combination. At indicated times (A) or at 72 h (B), cells were harvested and processed for either TUNEL assay (A) or propidium iodide staining (B) to determine the percentage of apoptotic cells (A) or the percentage of cells in sub-G₁ (B).

somes, allowing cell killing at 10-fold-lower concentration. The combined treatment by a mitotoxin with adenoviruses provides a promising strategy in the development of reduced toxicity for effective cancer therapy.

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