Objective: To determine whether transducible peptides that inhibit the oncoproteins HDM2 and Bcl-2 may selectively kill uveal melanoma and retinoblastoma cells.

Methods: Peptides were tested by viability assay, flow cytometry, TUNEL (terminal deoxynucleotidyl transferase–mediated fluorescein-dUTP nick-end labeling) assay, Western blot analysis, and reverse transcription–polymerase chain reaction in cultured eye tumor cells and normal cells. Preclinical studies were performed in a rabbit xenograft model of retinoblastoma.

Main Outcome Measures: Cell survival, apoptosis, gene expression, and tumor regression.

Results: The anti–Bcl-2 peptide induced apoptosis in tumor cells, but it also caused apoptosis in normal cells in culture and induced retinal damage after intravitreal injection. In contrast, the anti-HDM2 peptide induced rapid accumulation of p53, activation of apoptotic genes, preferential killing of tumor cells, and minimal retinal damage after intravitreal injection. The anti-HDM2 peptide also induced regression of human retinoblastoma cells in rabbit eyes.

Conclusions: Peptide transduction is a promising new approach to molecular eye cancer therapy. Inhibition of HDM2 can selectively activate p53 in transformed cells and may be an effective strategy for inducing apoptosis in eye cancer cells with minimal damage to normal ocular tissues.

Clinical Relevance: Molecular characteristics of uveal melanoma and retinoblastoma may be used to design novel therapeutic agents that have greater specificity and fewer adverse effects than current therapies.

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UVEAL MELANOMA and retinoblastoma are the most common primary intraocular cancers in adults and children, respectively. Current treatments for eye cancer, such as radiation and chemotherapy, are effective in many patients. However, the mechanisms of action of these modalities are nonspecific, leading to substantial injury to normal tissues and consequent dose-limiting complications. Molecular therapy may reduce the complications of conventional therapies by antagonizing specific molecules in cancer cells and selectively killing tumor cells with less damage to normal cells.

Several oncoproteins have been identified as potential targets for therapeutic inhibition, including HDM2 and Bcl-2. HDM2 inhibits p53 and maintains it at very low levels in normal cells. However, some cancers, such as uveal melanoma, seem to functionally inhibit p53 by overexpressing HDM2. Because p53 is rarely mutated in uveal melanoma, inhibition of HDM2 may trigger apoptosis in uveal melanoma cells by releasing the endogenous, wild-type p53 from its negative interaction with HDM2. Bcl-2 blocks apoptosis by inhibiting pro-apoptotic proteins such as Bax. Bcl-2 is overexpressed in most uveal melanomas and is the most consistently observed molecular abnormality in this eye cancer. Thus, inhibition of Bcl-2 may liberate pro-apoptotic proteins and allow them to trigger apoptosis in uveal melanoma cells.

Although HDM2 and Bcl-2 have not been adequately studied in retinoblastoma, inhibition of these proteins may also be effective in this pediatric eye cancer, which usually expresses wild-type p53 and is susceptible to apoptosis.

Transducible peptides represent a promising new technology for efficiently delivering designer therapeutic molecules into cells. Transducible peptides contain a therapeutic domain linked to a transduction domain that allows the...
chimeric peptide directly to traverse, or “transduce,” cell membranes independent of cell surface receptors or other transport molecules.13 Eye cancers provide an attractive model for testing transducible peptides because therapeutic agents can be delivered locally to the tumor using standard ophthalmic techniques. In this article, we present preliminary studies to examine transducible peptide inhibitors of HDM2 and Bcl-2 in cultured eye tumor cells and in a rabbit model of retinoblastoma.

METHODS

CULTURED CELLS

Primary uveal melanoma cells (MM-23, MM-24, and MM-26) were obtained from 3 patients and established in short-term culture (cells used after ≤3 passages). Cultures of normal choroidal fibroblasts and melanocytes (MM-23N and MM-26N) were obtained from 2 of these patients. Y79 and WERI-RB1 retinoblastoma cells, U2OS osteosarcoma cells, and C33A cervical carcinoma cells were obtained from American Type Culture Collection (Manassas, Va). Adult human diploid fibroblasts and mouse embryonic fibroblasts were obtained from BioWhittaker Inc (Walkersville, Md). Cells were maintained in Dulbecco Modified Eagle Medium with 0.1% or 10% fetal calf serum (as indicated) at 37°C in a humidified atmosphere of 5% CO2.

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis was performed as previously described. Briefly, we used the streptavidin-biotin method with the Vector ABC Elite kit (Vector Laboratories Inc, Burlingame, Calif) and nuclear fast red for counterstain. Four-micron sections of paraffin-embedded primary tumor tissue (MM-23, MM-24, and MM-26) or cytopsins of 105 cultured cells (U2OS, Y79, and WERI) were deparaffinized, rehydrated with alcohol, and treated with 0.3% hydrogen peroxide. Heat-induced antigen retrieval was performed. Antibodies against HDM2 (NCL-MDM2; Novocastra Laboratories Ltd, Newcastle, England), 1:30 dilution; p53 (clone 1801; Biogenics, Napa, Calif), 1:80 dilution; and Bcl-2, 0.5 µg (Dako, Glostrop, Denmark), 1:500 dilution, were applied at 4°C overnight. The percentage of positive cells for each antibody was estimated by counting at least 200 cells in at least 8 fields with magnification ×40 for each specimen.

PEPTIDES

Transducible peptides were generated by linking the Tat transduction sequence YGRKKRRQRRKG to the amino-terminus of the following peptides: Tat-p53 (a 12-amino acid peptide derived from the p53 sequence that mediates binding to HDM2), Tat-p53-HDM2-al (a 12-amino acid control mutant of the HDM2 binding peptide in which the residues required for binding to HDM2, corresponding to Phe-19 and Trp-23 in p53, are converted to alanine), Tat-p53H2 (a 19-amino acid peptide derived from the BH3 domain of the protein Bad, which binds and inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xL), Tat-scrambled (an 8-amino acid control peptide with a randomly scrambled sequence), dBcl2 (the Bcl-2 binding peptide without Tat), and Tat alone. Peptides were synthesized (John Gorka, PhD, Biomolecules Midwest, Inc, St Louis, Mo) using an ABI 431 synthesizer and eluted with 0.1N acetic acid. Amino acid content and purity greater than 90% were confirmed by mass spectrometry. Peptides were solubilized in sterile water and analyzed at a range of 50 to 300µM. The optimal ratio of tumor cell—normal cell killing was observed at 200µM, so most subsequent studies were performed at this concentration.

CELL STUDIES

Cell survival was measured by MTS assay (Promega Inc, Madison, Wis). Cells were seeded onto 96-well plates at 104 cells/well in Dulbecco Modified Eagle Medium with 10% fetal calf serum. Peptides were added to the media the next day, and spectrophotometric assay was performed 24 hours later. TUNEL (terminal deoxynucleotidyl transferase–mediated fluorescein-dUTP nick-end labeling) assay was performed 8 hours after addition of the indicated peptides using the Apoptag kit (Intergen Inc, Purchase, NY). The broad-spectrum caspase inhibitor Z-Val-Ala-Asp(OCH3)-fluoromethylketone (BIOMOL Research Laboratories Inc, Plymouth Meeting, Pa) was added to MTS plates as indicated. For Western blot analysis and reverse transcription–polymerase chain reaction (RT-PCR), cells were obtained at various points after the addition of 200µM Tat-p53HDM2, lysates were obtained, and protein concentration was normalized. Western blot analyses were performed using a polyclonal p33 antibody (sc-6243; Santa Cruz Biotechnology Inc, Santa Cruz, Calif), dilution 1:500, and a polyclonal p73 antibody (sc-7937; Santa Cruz Biotechnology), 1:500 dilution. For semiquantitative RT-PCR, total RNA was obtained using the RNAeasy kit (Qiagen, Valencia, Calif). Reverse transcription was performed in 20 µL of RNase-free water containing 5mM magnesium chloride, 10mM Tris-hydrochloride (pH 8.8), 50mM potassium chloride, 0.1% Triton X-100, 1mM dNTP, 20 U of rRNase inhibitor, 15 U of AMV reverse transcriptase (Promega Inc), and 0.5 µg oligo(dT)15 nucleotide primers at 42°C for 60 minutes, 99°C for 5 minutes, and then 5°C for 5 minutes. For PCR, 5 µL of the reverse transcription product was added to a 25-µL mixture containing primers for p21, Bax, PGP, or GAPDH, and 2.5 U of Taq polymerase in standard PCR buffer (GIBCO BRL, Grand Island, NY). Reaction cycles (n=20-25) and temperatures varied for each gene (details and primer sequences are available from the authors on request).

ANIMAL EXPERIMENTS

New Zealand White rabbits (2-4 kg) were used. For toxicity studies, peptides were injected into the vitreous cavity, and eyes were obtained 48 hours later for histopathologic examination. To create intraocular tumors, 105 WERI cells were injected into the right anterior chambers of animals immunosuppressed with cyclosporine (15 mg/kg per day). Tumors were observed daily by visual inspection of the anterior chamber, and they were observed to implant and begin growing within 1 to 2 weeks. Peptides were dissolved in sterile water and injected into the anterior chambers at an estimated intraocular concentration of 200µM. Two injections were administered over 3 days, and animals were humanely killed 48 hours later. Eyes were embedded in paraffin, stained with hematoxylin-eosin, or analyzed for apoptosis using the Apoptag TUNEL kit. Tumor destruction was quantified by projecting histopathologic sections and scoring areas of viable and apoptotic tumor. Studies conformed to the animal studies committee protocol of Washington University.

RESULTS

Amino acid sequences for peptides used in this study are listed in Table 1. The immunohistochemical expression of p53, HDM2, and Bcl-2 for each tumor sample is
summarized in Table 2. Transducible peptides tagged with fluorescein were shown to enter approximately 100% of cells and cell nuclei within 1 to 2 minutes of addition to culture media (data not shown), as previously described.15

INHIBITION OF Bcl-2

Tat-αBcl2 efficiently induced cell death in MM-23, MM-24, MM-26, Y79, WERI, and U2OS cells (Figure 1 and Figure 2A). However, Tat-αBcl2 also induced variable levels of cell death in normal primary choroidal cells (MM-23N and MM-26N), mouse embryonic fibroblasts, and normal human diploid fibroblasts (Figure 2A). Intravitreal injection of Tat-αBcl2 in normal rabbit eyes (approximate intraocular concentration, 100µM) caused mild to moderate disruption of retinal cells, especially in the inner nuclear layer (Figure 2B). Approximately 75% of the apoptosis induced by Tat-αBcl2 was blocked by the caspase inhibitor Z-Val-Ala-Asp(OCH₃)-fluoromethylketone (Figure 3A).

INHIBITION OF HDM2

Tat-αHDM2 efficiently killed MM-23, MM-24, MM-26, Y79, WERI, U2OS, and C33A cells (Figures 1 and 2A). Tat-αHDM2 induced positive TUNEL staining in tumor cells consistent with apoptosis (Figure 3B). At concentrations that induced apoptosis in tumor cells, Tat-αHDM2 had little effect on normal cells (Figure 2A and Figure 4). Intravitreal injection of Tat-αHDM2 in normal rabbit eyes (approximate intraocular concentration, 200µM) induced minimal histologic damage to the

<table>
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<th>Tumor</th>
<th>p53*</th>
<th>HDM2</th>
<th>Bcl-2</th>
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<tr>
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*Low p53 expression (<20%) suggests absence of mutation.
retina and other ocular tissues (Figure 2B). Treatment of cells with Z-Val-Ala-Asp(OC\(_3\))-fluoromethylketone blocked approximately 65% of the apoptosis induced by Tat-\(\alpha\)HDM2 (Figure 3A). In U2OS cells, which express low levels of wild-type p53 due to overexpression of HDM2, Tat-\(\alpha\)HDM2 caused a rapid increase in p53 protein levels, which remained high for 6 hours after treatment. Tat-\(\alpha\)HDM2 also caused an increase in p73 protein levels (Figure 3C). By semiquantitative RT-PCR, Tat-\(\alpha\)HDM2 activated the p53 target genes p21, Pig3, and Bax, which reached peak protein levels 4 to 6 hours after treatment (Figure 3C).

An animal model of intraocular retinoblastoma was created by injecting 10\(^7\) WERI human retinoblastoma cells into the anterior chambers of rabbit eyes and allowing the tumors to implant and grow (Figure 5A). After injection of Tat-\(\alpha\)HDM2 (approximate intraocular concentration, 200\(\mu\)M), tumors began to dissolve into a fine cloud within 24 hours. In contrast, tumors treated with the Tat-scrambled control peptide remained unchanged from their pretreatment appearance. Peptides were injected again 24 hours later, and the eyes were obtained. By histopathologic examination, Tat-\(\alpha\)HDM2 caused extensive tumor destruction, with a 76% reduction in viable tumor mass compared with tumors treated with the control peptide (Figure 5B-E). A much larger proportion of tumor cells were positive for TUNEL staining in eyes treated with Tat-\(\alpha\)HDM2 compared with eyes treated with the control peptide (Figure 5F and G). No histologic damage to the cornea, lens, retina, or other ocular tissues was detected.

**COMMENT**

In this study, we tested transducible peptides that inhibit HDM2 and Bcl-2 for their ability to induce tumor-specific apoptosis in uveal melanoma and retinoblastoma cells. An anti-Bcl-2 peptide induced apoptosis in tumor cells but also caused variable levels of toxicity in normal cells and tissues. In contrast, an anti-HDM2 peptide induced apoptosis in tumor cells, with little effect on normal cells in a therapeutic dose range. This peptide also caused regression of retinoblastoma in rabbit eyes, with minimal damage to normal ocular tissues. We conclude that inhibition of HDM2...
may be a promising strategy for the treatment of uveal melanoma and retinoblastoma and that transducible peptides may be an effective technology for local delivery of anti-cancer therapy to the eye.

Since Bcl-2 is frequently overexpressed in uveal melanomas and is thought to protect tumor cells from cell death, we tested whether inhibition of Bcl-2 might lead to apoptosis in uveal melanoma cells. For these experiments, we generated the peptide Tat-αBcl2, which is derived from the BH3 domain of the pro-apoptotic protein Bad, which has been shown to bind and inhibit the anti-apoptotic proteins Bcl-2 and Bcl-XL. Tat-αBcl2 efficiently induced apoptosis in each of the uveal melanoma cell lines, which were all derived from tumors that overexpressed Bcl-2 (Table 2). This peptide induced apoptosis that was largely caspase dependent, consistent with activation of the Bcl-2 mitochondrial apoptotic pathway. Tat-αBcl2 also killed U2OS osteosarcoma cells and Y79 and WERI retinoblastoma cells, although Bcl-2 was not overexpressed in these cells (Table 2). In addition, the peptide had a moderate cytotoxic effect on normal cells, suggesting that inhibition of Bcl-2 may trigger apoptosis in a broad spectrum of cells by destabilizing the delicate balance between pro-apoptotic and anti-apoptotic proteins in the Bcl-2 pathway. Activation of this pathway, which is downstream of the p53 tumor suppressor checkpoint, may then lead to generalized activation of the apoptotic program. Therefore, the anti-Bcl-2 peptide did not demonstrate a significant therapeutic window and was not studied further.

HDM2 binds and inhibits p53, maintaining it at low concentrations in normal cells. In cells that sustain oncogenic mutations, the HDM2-p53 interaction is disrupted, allowing p53 to accumulate, become activated, and induce growth arrest or apoptosis. Therefore, most tumors develop strategies for circumventing this tumor suppressor checkpoint, such as by mutating p53 or overexpressing HDM2. Because HDM2 is strongly expressed in most uveal melanomas and may serve to func-
studies provide an initial framework for future studies to determine appropriate molecular targets, optimal routes of administration, and indications for and limitations of transducible peptide therapy for eye cancers.

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