Activating Transcription Factor 2-derived Peptides Alter Resistance of Human Tumor Cell Lines to Ultraviolet Irradiation and Chemical Treatment

Anindita Bhoumik, Vladimir Ivanov, and Ze’ev Ronai

Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029

ABSTRACT

Activating transcription factor 2 (ATF2) and its kinase, p38, play an important role in the resistance of melanoma to radiation and chemotherapy. Whereas ATF2 up-regulates the expression of tumor necrosis factor α, which serves as a survival factor in late-stage melanoma cells, p38 attenuates Fas expression via inhibition of nuclear factor-κB. We investigated whether ATF2-derived peptides could be used to alter the sensitivity of human melanoma cells to radiation and chemical treatment. Of four 50-amino acid peptides tested, the peptide spanning amino acids 50-100 elicited the most efficient increase in the sensitivity of human melanoma cells to UV radiation or treatment by mitomycin C, Adriamycin, and verapamil, or UCN-01, as revealed by apoptosis assays. Sensitization by ATF2 peptide was also observed in the MCF7 human breast cancer cells but not in early-stage melanoma or melanocytes, or in in vitro-transformed 293T cells. When combined with an inhibitor of p38 catalytic activity, cells expressing amino acids 50-100 of ATF2 exhibited an increase in the degree of programmed cell death, indicating that combined targeting of ATF2 and p38 kinases is sufficient to induce apoptosis in late-stage melanoma cells. The ability of the peptide to increase apoptosis coincided with increased cell surface expression of Fas, which is the primary death-signaling cascade in these late-stage melanoma cells. Overall, our studies identified a critical domain of ATF2 that may be used to sensitize tumor cells to radiation and chemical treatment-induced apoptosis and that can induce apoptosis when combined with inhibition of ATF2 kinase, p38.

INTRODUCTION

The hallmark of malignant melanoma is its poor response to chemo- and radiotherapy. Despite advances in understanding of the biology of this tumor type (1), the nature of melanoma’s protection against radiation-induced apoptosis remains largely unknown. The ability to resist apoptosis by rearranging the apoptosis machinery, including Fas, TNF receptor 1, death receptor 3, and TNF-related apoptosis-inducing ligand receptor 1 and 2 (2, 3), is characteristic of most tumor cells, including melanomas (4). Altered susceptibility to apoptosis was shown to include suppression of the death receptor or increased expression of inhibitory apoptosis proteins that restrain caspase activity (5). Common to late-stage melanoma cells is the expression of a large subset of growth factors, cytokines and their respective receptors, which contribute to autocrine and paracrine regulation of their progression (6). Among the latter are TNFα-TNF receptor 1 and Fas-FasL, whose interaction elicits either death- or survival-signaling cascades. These cascades are regulated by the signal adapter TRAF2 and its downstream effectors, i.e., stress-activated kinases and their respective transcription factors (7-9).

Key signaling molecules documented to play an important role in the biology of melanoma consist of cell adhesion molecules, including cadherins, integrins, MUC18, intercellular adhesion molecule (10), MHC class I (11), PTEN and PI3K (12-14), the Ras oncogene (15), the stress kinases JNK and p38 (16, 17), and their upstream regulator TRAF2 (18), as well as signal-transducing molecules, including β-catenin (19-21), and cell cycle regulators such as p16 (22). Either mutation or altered expression has been reported for these regulatory proteins, which confers the changes implicated in the development and progression of human melanoma.

In comparing early- and late-stage melanoma cells, we identified lower expression and activities of TRAF2 and its respective effectors GCK and NF-κB in early-stage melanoma cells (18). Low expression levels of TRAF2/GCK in early-stage melanoma cells coincide with low level of c-Jun and NF-κB activities. Forced expression of GCK in these cells efficiently increased the resistance of the early-phase melanoma to radiation. Similarly, expression of the dominant-negative form of GCK in late-stage melanoma reduced the resistance of late-phase melanoma cells lines to radiation (18). These observations

Received 8/18/00; revised 11/7/00; accepted 11/8/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Z. R. was the recipient of Grant CA 51995 from the National Cancer Institute.
2To whom requests for reprints should be addressed, at The Ruttenberg Cancer Center, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1130, New York, NY 10029. Fax: (212) 849-2425; E-mail: zeev.ronai@mssm.edu.

3 The abbreviations used are: TNF, tumor necrosis factor; TRAF2, TNF receptor-associated factor 2; PI3K, phospho-inositol 3 kinase; JNK, Jun NH₂-terminal kinase; GCK, germinal center kinase; NF-κB, nuclear factor-κB; ATF2, activating transcription factor 2; FBS, fetal bovine serum; JAK, Janus-activated kinase; MMC, mitomycin C; NCS, necarzinostatin; FACS, fluorescence-activated cell sorting; CFE, colony-forming efficiency.
pointed to changes in the regulation of key stress signaling molecules during melanoma progression and to the role of TRAF2 and its effector GCK in acquiring radiation resistance of late-phase human melanoma cells.

In elucidating transcription factors that may alter the resistance of melanoma to UV irradiation, we identified cAMP-responsive element binding protein-associated proteins (23), among which ATF2 was found to play an important role in acquiring such resistance (24). Hypophosphorylated or transcriptionally inactive forms of ATF2 elicit a silencing effect on TNFα expression, which mediates an antiapoptotic signal in LU1205, a late-stage melanoma cell line, resulting in increased apoptosis (25). The importance of the p38 signaling cascade, which is among major ATF2 kinases, in the biology of human melanoma was further demonstrated by the finding that p38 negatively regulates the expression of Fas via suppression of NF-κB transcriptional activity (17). Thus, p38 appears to play a key role in the ability of melanoma to acquire resistance to radiation-induced apoptosis through its ATF2 effector, which up-regulates TNFα expression, and via direct p38 suppression of NF-κB, which down-regulates Fas expression. On the basis of these findings, the current study aimed at assessing the ability to sensitize melanoma cells to apoptosis by outcompeting endogenous ATF2 expression with ATF2-derived peptide(s) alone and in combination with inhibition of p38 activities via its pharmacological inhibitor. We demonstrate that expression of a 50-amino acid peptide derived from the NH2-terminal domain of ATF2 is sufficient to sensitize melanoma as well as breast cancer cells to radiation and chemical treatment, and that the combination of this peptide with the pharmacological inhibitor of p38 is sufficient to induce programmed cell death in late-stage melanoma cells that use Fas as a major death-signaling cascade.

MATERIALS AND METHODS

Cell Lines. LU1205 cells (also known as 1205LU), a late-stage human melanoma cell line, were maintained in MCD8131/L15 medium (4:1) supplemented with 5% FBS, l-glutamine, and antibiotics. LU1205 cells that stably expressed ATF2-derived peptide II or peptide IV were maintained in the same medium supplemented with G418 (200 μg/ml). The late-stage melanoma cells, FEMX (kind gift of Dr. Ø. Fodstad) were maintained in RPMI supplemented with 10% FBS and antibiotics. The medium for WM1552 cells, an early-phase human melanoma cell line, was the same as for the LU1205 cells (both were kindly provided by Dr. M. Herlyn), supplemented with insulin (5 μg/ml). Human melanocytes (FOM71; kind gift of Dr M. Herlyn) were maintained in MCDB151 (Sigma) medium supplemented with 2% FBS (Cansera), 10% chelated FBS (Sigma), 2 mM l-glutamine (Cellgro), 20 μM cholaer toxin (Sigma), 100 μM endothelin 3 (Peninsula), 10 ng/ml stem cell factor (Research & Development), and 250 pg basic fibroblast growth factor (Life Technologies).

Chemicals. The pharmacological inhibitors of JAKs (AG490), p38 (SB203580), and PI3K (LY294002) were purchased (Calbiochem). MMC, Adriamycin, and verapamil were purchased from Sigma. The radiomimetic drug NCS was obtained from Kayaku Co. (Tokyo, Japan). The nuclear export inhibitor leptomycin B was kind gift of Dr. Yoshida (Kyushu University, Japan). The chemotherapeutic drug 7-hydroxystaurosorpine (UCN-01) was kindly provided by the Drug Synthesis and Chemistry Branch at the National Cancer Institute.

Stable Transfection and Selection. Oligonucleotides corresponding to ATF2 peptides within amino acids 1–50 (peptide I), 50–100 (peptide II), 100–150 (peptide III), and 150–200 (peptide IV) were PCR amplified and cloned into BamHI and Xhol sites of pcDNA3 (Invitrogen, Carlsbad, CA), which contained a HA-penetratin tag on its NH2-terminal domain. Cloned material was verified via sequencing. pcDNA3-HA-neo or pcDNA3-HA encoding each of the four peptides was electroporated (230 V, 1050 microfarads) into the respective cell lines as described previously (24). Cells were maintained in G418 (500 μg/ml) for 2 weeks before mixed populations were pooled and characterized.

Immunohistochemistry and Western Blot Analysis. Cells were grown on coverslips before being subjected to fixation with PBS containing 3% paraformaldehyde and 2% sucrose (10 min at room temperature), followed by permeabilization with PBS containing 0.5% Triton X-100, 3 mM MgCl2, and 6% sucrose (5 min on ice). Cells were then incubated with antibodies against HA-tag (5 μg/ml) for 1 h at 20°C, before being washed with PBS and incubated with secondary (antimouse IgG) antibody, which was conjugated to FITC (Roche Chemicals), for 1 h at 20°C. Immunofluorescence analysis was carried out using a fluorescence microscope (Nikon). Western analysis for expression of the low molecular weight peptides was carried out using 15% Tricine SDS-PAGE and antibodies to HA. Secondary antibodies used in this reaction were goat antimouse IgG conjugated to horseradish peroxidase (1:500). Signals were detected using the ECL system (Amersham-Pharmacia Biotech).

Treatment and Apoptosis Studies of Stably Transfected Melanoma Cells. Cells were exposed to UV-C irradiation at 75 J/m2 as described previously (24). SB203580 (1–10 μM; Calbiochem, San Diego, CA), NCS (50–100 ng/ml), and MMC (0.2–1 μM) were used to treat melanoma cells. Cells were pelleted and resuspended in 0.5 ml of hypotonic buffer with 0.1% Triton X-100 containing propidium iodide (40 μg/ml) and DNase-free RNase A (1 mg/ml). Cells were incubated at 37°C for 30 min and analyzed on a Calibur flow cytometer (Becton Dickinson) using CellQuest software as described previously (26). The percentage of cells to the left of the diploid G0/G1 peak, characteristic of hypodiploid cells that have lost DNA, was defined as the percentage of apoptotic cells. Analysis was performed with light scatter gating. Surface expression of Fas was determined using anti-Fas-phycoerythrin antibody (PharMingen CA) and flow cytometric analysis. Cell surface expression is measured as mean fluorescence intensity.

Radiation Resistance. Cells (500 or 1500 per well) were plated in triplicate on 6-well plates, 24 h before treatment (24). In all cases, plating efficiency was predetermined so that number of cell plated was normalized. Colonies (>50 cells per clone) were stained with crystal violet solution (3% in 10% methanol and PBS) 14 days after treatment. The percentage of CFE in each treatment group was calculated based on total CFE in the respective controls (100%).
RESULTS

Expression of ATF2-derived Peptides in Melanoma Cells. The four peptides selected for the analysis span the first 200 amino acids of ATF2. We focused on this domain, which represents the transactivating domain, because a form of ATF2 that lacked this region efficiently decreased the resistance of melanoma to radiation treatment (24). We thus hypothesized that expression of a peptide from the transactivating domain may efficiently out compete some of the ATF2 modifications required for its transcriptional activities. Among important sites within the first 200 amino acids are the phosphoacceptor sites for p38 and JNK (amino acids 69 and 71; Refs. 27) and the region required for ATF2 intramolecular inhibition (within amino acids 150–200; Ref. 28).

Clones of the FEMX and LU1205 melanoma cells expressing the respective peptides were selected and maintained as mixed populations. To verify expression of the peptides, mRNA was used for reverse transcription-PCR using primers against each of the corresponding peptides. PCRs confirmed the expression of the respective peptides (not shown). To determine expression at the protein level, immunohistochemistry was carried out using antibodies to the HA-tag. As shown in Fig. 1A, expression of peptide II or peptide IV was clearly observed, albeit primarily in the cytoplasm. More than 80% of the cells were found to express these peptides. To determine whether the peptides could also be found in the nuclear fraction, cells were treated with the nuclear export inhibitor, leptomycin B, which enabled detection of the peptides in the nuclei (Fig. 1B). Western blot analysis using antibodies directed to the HA-tag further confirmed their expression (Fig. 1C). These results demonstrate that melanoma cells express the ATF2-derived HA-tagged peptides in both the nuclear and the cytoplasmic cellular compartments. Because these peptides were derived from the ATF2-transactivating domain, we monitored possible changes to ATF2 expression and phosphorylation in cells that constitutively express these peptides. As shown in Fig. 1D, melanoma cells that expressed peptide II exhibited a higher degree of ATF2 phosphorylation under nonstressed growth conditions but not after exposure to UV irradiation. Conversely, peptide IV-expressing cells exhibited a larger increase in ATF2 phosphorylation after UV treatment compared with the parent (control vector-expressing) cells (Fig. 1D). These observations suggest that expression of peptide IV, but not peptide II, may further induce the transcriptional activities of endogenous ATF2.

Effect of ATF2 Peptides on Radiation Resistance of Human Melanoma Cells. To determine the effect of each of the four peptides on the radiation resistance of the LU1205 and FEMX cells, two late-phase melanoma-derived cell lines that were selected based on their high resistance to radiation,4 we

---

4 V. Ivanov and Z. Ronai, unpublished observations.
used the classical radiation resistance assay, namely, colony-forming efficiency. Cells plated to assure equal numbers were subjected to irradiation using UV-C as the source. Colonies numbering >50 cells/clone were counted 14 days later. These analyses, carried out in triplicate, revealed that two of the four peptides induced clear changes in the sensitivity of melanoma cell lines to UV treatment. Whereas the expression of peptide IV led to an increase in resistance to UV irradiation, peptide II sensitized both LU1205 and FEMX melanoma cells to UV treatment (Fig. 2). Peptide II decreased the percentage of CFE of LU1205 cells up to 10-fold (Fig. 2A), whereas peptide IV increased the percentage of CFE of both the LU1205 and FEMX melanoma cell lines up to 2.5-fold in response to UV treatment (Fig. 2B).

These observations suggest that peptide II efficiently decreased the resistance of melanoma cells to irradiation, whereas peptide IV had the opposite effect. Because these two peptides exhibited the most pronounced effects, they were selected for additional characterization.

**Effect of ATF2 Peptides on Resistance of Melanoma to Chemical Treatment.** To determine whether the effects of ATF2-derived peptides extend beyond UV irradiation, we subjected the melanoma cells to treatment with chemicals often used for chemotherapy. Treatment of FEMX cells that expressed peptide II with 1 μM MMC decreased the fraction of CFE up to 10-fold compared with the control FEMX neo-expressing cells (Fig. 3A; 1 μM dose). Peptide II had much less pronounced effects on the sensitivity of LU1205 to MMC. Conversely, peptide IV was as efficient in increasing (4–5-fold) the resistance of both melanoma cell lines to MMC treatment (Fig. 3B). These results suggest that ATF2-derived peptides could also alter the sensitivity of selective melanoma cells to MMC.

Treatment with the chemical NCS, which mimics the effects of X-ray, did not reveal substantial differences in the sensitivity of melanoma cells expressing ATF2-derived peptide II (Fig. 3C). Peptide IV produced an almost 2-fold increase in the resistance to low doses of NCS (Fig. 3C). These results suggest that ATF2 peptides are not effective in sensitizing melanoma cells to the radiomimetic drug NCS. Indeed, ATF2 peptide-expressing melanoma cells did not exhibit changes in the sensitivity to X-ray treatment (data not shown), suggesting that a different signaling pathway is used by melanoma to acquire resistance to X-ray.

Control and peptide-expressing cultures were also subjected to treatment with commonly used chemotherapeutic drug Adriamycin alone or in combination with verapamil, which is used to avoid induction of drug resistance. As shown in Fig. 3D, sensitivity of LU1205 cells to Adriamycin-induced programmed cell death increased in response to Adriamycin treatment (2-fold compared with control). The combination of Adriamycin and verapamil caused a 4-fold increase in apoptosis of control cells, and an additional (50%) increase in peptide II-expressing cells (10-fold compared with neo-expressing cells). Of interest, peptide IV-expressing LU1205 cells exhibited a 70% increase in the degree of apoptosis over the control neo-expressing cells subjected to the combination of Adriamycin and verapamil (Fig. 3D). These observations suggest that the effects mediated by ATF2-peptides are selective to the form of DNA damage and stress.

**ATF2 Peptides Affect UV-induced Apoptosis of Human Melanoma Cells.** Previous studies showed that the mechanism underlying the ability of ATF2 to alter the resistance of melanoma to radiation involved changes in the melanoma cell’s ability to undergo programmed cell death (24). For this reason, we determined whether the ATF2-derived peptides are capable of altering the sensitivity of the melanoma cell to apoptosis. FACs analysis of the melanoma cells carried out 36 h after irradiation clearly revealed changes in the percentage of cells that had undergone programmed cell death. Peptide II caused an increase (from 15% to 38%) in the apoptotic fraction of UV-treated LU1205 cells (Fig. 4A), and a corresponding increase (from 13% to 28%) was seen in UV-treated FEMX cells that expressed this peptide (Fig. 4B). Conversely, peptide IV decreased the fraction of cells that underwent apoptosis in both LU1205 cells (from 15% to 10%; Fig. 4A) and FEMX cells (from 13 to 8%; Fig. 4B). These observations suggest that the changes in resistance to irradiation caused by the two peptides
are mediated by alterations in the melanoma cells' sensitivity to apoptosis in response to UV irradiation.

We also assessed the effect of ATF2-derived peptides on UV-induced apoptosis in the early-phase WM1552 melanoma cell line. Previous studies had disclosed that WM1552 early-phase melanoma cells are more sensitive to irradiation than late-stage melanoma cells as a result of impaired signaling by TRAF2/GCK, which affects the activities of both the JNK and the NF-κB pathways (18). In contrast to its effects on the LU1205 and FEMX late-stage melanoma cells, peptide II no longer was able to elicit sensitization of these cells to UV effects (compare control and peptide-expressing WM1552 cells in Fig. 4).
Nevertheless, both peptides caused a noticeable (4–5-fold) increase in the basal level of apoptosis of these early-phase melanoma cells (Fig. 4C). These results imply that the ability of peptide II to increase the sensitivity of melanoma cells to irradiation may depend on the nature of the stress signaling cascade, which is different in the early-phase melanoma cells (weak JNK and NF-kB activities compared with late-stage LU1205; Ref. 18). UV treatment of peptide IV-expressing WM1552 cells did not cause higher level of apoptosis when compared with nontreated WM1552 cells expressing this peptide (Fig. 4C), suggesting that expression of this peptide attenuated the ability of UV irradiation to induce cell death.

**ATF2 Peptides Alter Sensitivity to UV Radiation in Non-melanoma Tumor-derived Cell Lines.** An important consideration in our analysis of ATF2-derived peptides was to determine whether similar effects could be elicited in non-melanoma tumors. To this end, different tumor-derived cell lines were transfected with peptide II or peptide IV, and stably expressing cells were then used for analysis.

Expression of peptide II in the MCF7 breast cancer-derived cell line caused a minimal effect on the basal level of apoptosis. Nevertheless, whereas neo-resistant parent MCF7 cells (used as a control) exhibited a 4-fold increase in apoptosis after UV treatment, the peptide II-expressing cells exhibited a close to 9-fold increase in apoptosis relative to nonirradiated cells (Fig. 5A). Opposite to the effect of peptide II, peptide IV caused a decrease in apoptosis after UV treatment (5-fold compared with MCF7 neo control cells; Fig. 5A). These findings establish that ATF2-derived peptides are also capable of altering the sensitivity of breast cancer cells to UV treatment.

MCF7 is among the better-characterized breast cancer cell lines, which has led to the development of a battery of MCF7 derivatives selected for growth based on their ability to develop drug resistance. One such MCF7 derivative is the Adriamycin-resistant MCF7 cell line, which is 1000 times more resistant to Adriamycin than the original cell line. Peptide II-expressing MCF7-Adr cells exhibited an 8-fold increase in apoptosis after UV treatment compared with the 3-fold increase in the MCF7-Adr-neo controls (Fig. 5B). This result suggests that peptide II retained its ability to sensitize cells to UV treatment even if they have acquired resistance to chemotherapeutic drugs. Of interest was the finding with peptide IV, which no longer was able to increase the resistance of MCF7 cells to UV treatment implies that Adriamycin and peptide IV may use similar cellular pathways to increase resistance of tumor cells to radiation and chemical treatments; since these cells had already acquired chemotherapeutic resistance, these pathways were no longer affected by peptide IV. Of interest is that the basal level of apoptosis, which was affected in the MCF7 cells by both ATF2-derived peptides (Fig. 5A), was no longer seen in the MCF-Adr cells (Fig. 5B), further pointing to changes in MCF7-Adr that impaired the contribution of the ATF2 pathway to basal levels of programmed cell death.

Unlike its effect in the breast cancer-derived cell lines, peptide II was not able to increase the sensitivity of in vitro-transformed 293T human kidney embryonic cells to irradiation. Nevertheless, as also seen with MCF7 and WM1552 cells, both peptides were able to efficiently increase the basal level of apoptosis. Whereas peptide II caused a 5-fold increase, peptide IV led to a 15-fold increase in the basal level of apoptosis seen in 293T cells that were maintained under normal growth conditions (Fig. 5C). Furthermore, peptide IV efficiently increased the basal level of apoptosis in nonstressed cells.

**ATF2 Peptides Increase Sensitivity of Melanoma Tumor-derived Cell Lines to Chemotherapeutic Drug UCN-01.** To further explore the possible sensitization of melanoma cells to apoptosis by relevant chemotherapeutic drugs, we chose to test the effect of UCN-01. UCN-01 is a protein kinase inhibitor,
ATF2 peptides sensitize human melanoma and breast cancer cells to the chemotherapeutic chemical UCN-01. The late-stage human melanoma cell lines LU1205 (A) and FEMX (B) as well as the breast cancer cell line MCF7 (C) that stably expressed control vector (neo) or ATF2 peptides as indicated, were treated with the chemotherapeutic drug UCN-01 at the indicated concentrations, and the degree of apoptosis, measured by FACS analysis, was determined 12–18 h later. Data shown are from three different experiments.
presently undergoing clinical trials for cancer treatment, that abrogates the G2 checkpoint function via targeting of the Chk1 kinase and the Cdc25C pathways and that sensitizes p53-defective cancer cells to DNA-damaging agents (29–32). Thirty percent of LU1205 cells underwent apoptosis in response to a 1 μM dose of UCN-01. Expression of peptide II further increased the fraction of cells undergoing apoptosis to 40%, whereas expression of peptide IV somewhat decreased the degree of cell death (to 21%; Fig. 6A). Higher doses of UCN-01 caused up to 48% apoptosis in peptide II-expressing LU1205 cells compared with the 36% seen in the parent cells. A substantially greater sensitization was seen in the FEMX cells. A >3-fold increase in the degree of UCN-01-elicited apoptosis was seen in FEMX cells that expressed peptide II (from 10% to 36% at the 1 μM dose, and from 18% to 54% at the 5 μM dose) and to a lesser extent in FEMX cells that expressed peptide IV (Fig. 6B). These results suggest that peptide II is capable of sensitizing melanoma cells to the chemotherapeutic drug UCN-01 and that the degree of sensitization varies among the different melanoma tumor-derived cells. Further assessment of sensitization to UCN-01 was carried out in the breast cancer cell line MCF7. MCF7 cells that expressed peptide II exhibited a close to 2-fold increase in their sensitivity to UCN-01-elicited apoptosis (Fig. 6C). This increase was dose dependent: higher doses of UCN-01 further sensitized peptide II-expressing and, to a lesser degree, peptide IV-expressing MCF7 cells to apoptosis (Fig. 6C). Together, these finding establish that the expression of ATF2 peptides and in particular peptide II efficiently sensitizes melanoma and breast cancer cells to apoptosis induced by chemotherapeutic drugs, including MMC, Adriamycin + verapamil, and UCN-01.

**ATF2-derived Peptides Increase Fas Expression.** Our previous studies revealed that Fas is the primary apoptotic pathway in late-stage melanoma cells, which is balanced by the relative expression levels of TNFα (25). A decrease in TNFα or an increase in Fas results in elevated sensitivity to apoptosis. To determine the possible mechanism by which peptide II is capable of increasing the sensitivity of the human melanoma cell to UV-induced apoptosis, we followed changes in the cell surface expression of Fas. FACS analysis of LU1205 cells that expressed peptide II revealed a noticeable increase in their surface expression (mean fluorescence intensity increased from 122 to 186), whereas peptide IV did not cause major changes in cell surface expression of Fas (Fig. 7A). Analysis of Fas promoter activity using the −460 Fas
promoter region revealed an increase in Fas-mediated transcription in peptide II-expressing LU1205 cells, whereas peptide IV-expressing LU1205 cells exhibited a decrease in Fas promoter-mediated transcription (data not shown). Western blot analysis demonstrated an increase in the overall level of Fas protein in peptide II-expressing melanoma cells (Fig. 7B). The effects of ATF2-derived peptides on Fas expression point to the mechanism by which these peptides alter sensitivity of melanoma to UV and chemical treatments.

Inhibition of p38 Induces Apoptosis of Late-Stage LU1205 Melanoma Cells that Express Peptide II. p38 signaling of death pathways appears to use at least two independent cascades to affect melanoma resistance to radiation. The first has been attributed to the effect of the p38 substrate, ATF2, which up-regulates expression of TNFα (25), whereas the second relies on the ability of p38 to suppress NF-κB activities, resulting in suppression of Fas transcription (17). Given the dual activities elicited by p38, we sought to inhibit p38 catalytic activity in cells that already expressed ATF2-derived peptides. Treatment of the LU1205 cells expressing peptide II with SB203580, a pharmacological inhibitor of p38 (33), resulted in a dose-dependent increase in the degree of apoptosis (Fig. 8A). Whereas low doses of p38 inhibitor were sufficient to mediate a 3-fold increase in cell death, the higher doses (10 μM), which are likely to affect other kinases as well as p38 (i.e., JNK), led to an additional increase in the degree of cell death (>6-fold) in peptide II-expressing melanoma cells. This result suggests that in combination with peptide II expression, inhibition of stress kinase activities is sufficient to induce apoptosis in late-stage melanoma cells. The effects of p38 inhibitor and peptide II expression were additive, further supporting the notion that two p38-independent pathways were affected. Whereas treatment of LU1205 cells that expressed peptide IV with low concentrations of SB203580 did not alter the degree of cell death, at a higher concentration there was a 2-fold increase in the amount of cell death (Fig. 8A). These observations suggest that the ability of peptide IV to increase resistance of human melanoma cells to irradiation can be augmented by inhibition of stress kinases.

In contrast to the effect of the p38 inhibitor, treatment with LY294002, an inhibitor of PI3K, did not alter the sensitivity of either parent or ATF2 peptide-expressing melanoma cells to UV-induced apoptosis (Fig. 8B). Conversely, treatment with the JAK inhibitor (AG490) led to a 3-fold increase in the sensitivity of peptide II-expressing LU1205 cells to UV-induced apoptosis (Fig. 8B). This observation suggests that the ability of peptide II to sensitize melanoma cells to UV treatment may also involve members of the STAT family, which are JAK substrates.

Given the differences among the early- and late-stage melanoma cells tested here, we have further characterized whether ATF2 peptides could elicit changes in the sensitivity to apoptosis of normal melanocytes. Transient expression of peptide II did not cause a significant increase in the sensitivity of melanocytes to UV-induced apoptosis. In addition, unlike their effect in late-stage melanoma cells, neither NCS nor SB203580 elicited any significant changes in the degree of apoptosis in these melanocytes (Fig. 8C). These observations further support the
ATF2-derived Peptides Alter Melanoma Resistance

**DISCUSSION**

The present study has extended earlier observations in which ATF2 was identified as an important player in the ability of the melanoma cell to undergo apoptosis. Four 50-amino acid peptides obtained from the NH2-terminal domain of ATF2 were tested, of which two were selected for further characterization on the basis of their pronounced effect on late-stage melanoma cell lines. Of these two peptides, peptide II, which correspond to amino acids 50–100, efficiently increased the sensitivity of melanoma cells to UV irradiation as well as to MMC, Adriamycin + verapamil, and UCN-01 treatment. The effects of peptide II were as pronounced in the breast cancer cell line MCF7 and its derivative, MCF7-Adr, which is Adriamycin resistant, indicating that the effects studied here are not limited to melanoma cell lines and that peptide II may also sensitize Adriamycin-resistant breast cancer cells to DNA damage, illustrated here via UV treatment. Conversely, peptide II expression did not elicit changes in sensitivity to UV-induced apoptosis in 293T cells or in the early-phase WM1552 melanoma cells, nor was it effective in normal melanocytes. It is important to stress, however, that both ATF2 peptides had a pronounced effect on the basal level of apoptosis in both early melanoma (WM1552) and in vitro-transformed human 293T cells, suggesting that in these cells the role of ATF2 is more important in suppression of basal, rather than DNA damage-induced apoptosis. These differences also suggest that certain cellular components, which are shared among MCF7 and late-stage melanoma cells, are required for peptide II to elicit its effects in response to DNA damage. The noticeable differences in basal as well as UV-inducible apoptosis between early- and late-stage melanoma cells are likely attributable to altered TRAF2 expression, JNK activities mediated by activator protein 1 target sequences, which are regulated by the c-Jun-ATF2 heterodimers, are lower in melanoma cells that express peptide II (data not shown). Earlier studies from our laboratory revealed that phosphorylation-deficient full-length ATF2 has effects similar to those of the NH2-terminal truncated form, which serves as a dominant-negative; both effectively down-regulated expression of TNFα (24).

Interestingly, peptide IV, which bears amino acids 150–200 from ATF2, efficiently increased the sensitivity of tumor cells to UV irradiation and chemical treatment is likely to involve competition with the endogenous form of ATF2. Peptide II harbors amino acids 50–100 of ATF2, which contain the phosphorylation sites for the stress kinases p38 and JNK. It is possible that expression of ATF2 peptide decreases the phosphorylation of endogenous ATF2, thus rendering endogenous ATF2 inactive. Indeed, the level of ATF2 phosphorylation was reduced in UV-treated peptide II-expressing melanoma cells. Along those lines, the transcriptional activities mediated by activator protein 1 target sequences, which are regulated by the c-Jun-ATF2 heterodimers, are lower in melanoma cells that express peptide II (data not shown). Earlier studies from our laboratory revealed that phosphorylation-deficient full-length ATF2 has effects similar to those of the NH2-terminal truncated form, which serves as a dominant-negative; both effectively down-regulated expression of TNFα (24).

Peptide IV sensitized melanoma and breast cancer cells to UV- and drug-induced apoptosis. Conversely, peptide IV sensitized melanoma and breast cancer cells to X-ray radiation.
UCHN-01 treatment, albeit at lower efficiency than peptide II. Mechanistically, peptide IV is likely to interfere with the intrinsic inhibition of ATF2, which is mediated by intramolecular association between the COOH terminus of ATF2 and the NH₂-terminal zinc finger region. The inhibition can be disrupted by removal of amino acids 150–250, which renders ATF2 constitutively active. Indeed, a naturally occurring splice form variant of ATF2 is lacking amino acids 150–250 and is a constitutively active transcription factor (34). Interfering with the intramolecular inhibition of ATF2 is expected to result in a greater transcriptional output signal from the endogenous ATF2 protein. Indeed, peptide IV-expressing cells exhibited elevated transcriptional output as measured by Jun-2-Luc activities (data not shown).

With regard to the effect of ATF2 peptides, an intriguing observation was made with the p38 inhibitor SB203580, which caused a marked increase in the degree of apoptosis without additional exposure to DNA damage. This important observation confirms our recent studies in which we identified an independent cellular pathway by which p38 contributes to the resistance of human melanoma to UV-induced apoptosis, namely, inhibition of NF-κB activities and as a consequence, a decrease in Fas transcription (17). Thus, the combination of ATF2 competition together with inhibition of p38 catalytic activities is sufficient to cause a marked increase in the degree of apoptosis of late-stage melanoma cells.

Can a 50-amino acid peptide of a transcription factor be used as a source for pharmacomimetic drug design? Preliminary analysis of LU1205 cells that express peptide II, which were injected s.c. into nude mice and were subsequently subjected to SB203580 treatment revealed significant inhibition of tumor growth at the orthotopic site. Current studies are aimed at removing amino acids 150–250, which renders ATF2 constitutively active. Indeed, a naturally occurring splice form variant of ATF2 is lacking amino acids 150–250 and is a constitutively active transcription factor (34). Interfering with the intramolecular inhibition of ATF2 is expected to result in a greater transcriptional output signal from the endogenous ATF2 protein. Indeed, peptide IV-expressing cells exhibited elevated transcriptional output as measured by Jun-2-Luc activities (data not shown).

With regard to the effect of ATF2 peptides, an intriguing observation was made with the p38 inhibitor SB203580, which caused a marked increase in the degree of apoptosis without additional exposure to DNA damage. This important observation confirms our recent studies in which we identified an independent cellular pathway by which p38 contributes to the resistance of human melanoma to UV-induced apoptosis, namely, inhibition of NF-κB activities and as a consequence, a decrease in Fas transcription (17). Thus, the combination of ATF2 competition together with inhibition of p38 catalytic activities is sufficient to cause a marked increase in the degree of apoptosis of late-stage melanoma cells.

Can a 50-amino acid peptide of a transcription factor be used as a source for pharmacomimetic drug design? Preliminary analysis of LU1205 cells that express peptide II, which were injected s.c. into nude mice and were subsequently subjected to SB203580 treatment revealed significant inhibition of tumor growth at the orthotopic site. Current studies are aimed at reducing the size of peptide II to a level at which it would still be capable of eliciting efficient sensitization of late-stage melanoma cells to apoptosis induced by radiation and drug treatments. If this effort is successful, small-sized peptides may then be considered a source for pharmacomimetic drug design for treatment of melanoma.

ACKNOWLEDGMENTS

We thank Mark Bluth, X. Xu, and Karen Quadrini for technical assistance; Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) for LU1205, WM1552, and normal melanocytes; Dr. Ø. Fodstad (Oslo, Norway) for FEMX melanoma cells; Dr. M. Yoshida (Kyoto, Japan) for leptomycin B; and Kenneth Tew (Fox Chase Cancer Center, Philadelphia, PA) for the MCF7-Adr cells. We also thank Dr. Robert Schultz of the Drug Synthesis and Chemistry Branch at the National Cancer Institute (Bethesda, Maryland) for providing us with UCN-01.

REFERENCES

12. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., and Sellers, W. R. Regulation of G1 progression by the intramolecular inhibition of ATF2 is expected to result in a greater transcriptional output signal from the endogenous ATF2 protein. Indeed, peptide IV-expressing cells exhibited elevated transcriptional output as measured by Jun-2-Luc activities (data not shown).


Activating Transcription Factor 2-derived Peptides Alter Resistance of Human Tumor Cell Lines to Ultraviolet Irradiation and Chemical Treatment

Anindita Bhoumik, Vladimir Ivanov and Ze'ev Ronai


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/2/331

Cited articles
This article cites 33 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/2/331.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/7/2/331.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.