Inhibition of Homologue of Slimb (HOS) Function Sensitizes Human Melanoma Cells for Apoptosis

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Abstract

Homologue of Slimb (HOS)/β-transducin repeats containing proteins up-regulate nuclear factor κB activity by targeting its inhibitor (IκB) for ubiquitination and subsequent degradation. We investigated whether inhibition of HOS function may modulate apoptosis in human melanoma cells. Forced expression of the dominant negative HOSΔN construct inhibited IκB degradation and led to sensitization of melanoma cells to apoptosis induced by tumor necrosis factor α with cycloheximide, as well as by cisplatin and ionizing and UV irradiation. These data indicate that HOS plays an important role in controlling the IκB-dependent apoptotic pathways in human melanoma.

Introduction

Resistance of human malignant melanomas to radiation and commonly used chemotherapeutic agents is considered to be among the major clinical problems and reasons for poor prognosis of the metastatic disease (reviewed in Ref. 1). Because most anticancer agents kill susceptible cells via the induction of programmed cell death (apoptosis), the resistance of melanoma cells to apoptosis is a focus of studies aimed at clarifying the mechanisms underlying melanoma chemotherapeutic resistance. Recent studies have implicated NF-κB as a critical regulator of apoptosis. NF-κB is required for the regulation of survival genes (2–4) whose products can block cell death. NF-κB is a ubiquitous heterodimeric complex composed of p50 and p65/RelA subunits. This complex is normally sequestered in an inactive form in the cytoplasm through interaction with members of a family of inhibitory proteins, the IκBs. These proteins, when associated with NF-κB, mask the nuclear localization signal of p65 of the NF-κB complex and impair its ability to translocate to the nucleus and bind DNA (reviewed in Ref. 5). Sequential phosphorylation, ubiquitination, and proteasome-dependent degradation of IκB lead to the activation of transcription factor NF-κB in response to a variety of extracellular signals. IκB turnover seems to play a critical role in the regulation of NF-κB activities. Phosphorylation of IκBα at Ser32, Ser36 by IκB kinase marks IκBα for ubiquitination and proteasome-dependent degradation, thus allowing NF-κB nuclear translocation and target gene expression (reviewed in Ref. 6). Some melanoma cells exhibit elevated basal IκB kinase activity that results in an increased constitutive IκBα phosphorylation and degradation and NF-κB activation (7). Inhibition of NF-κB activities by nondegradable IκBα constructs promotes apoptosis in many cancer cells (including melanoma; Refs. 8–10). We have recently identified an F-box/WD40 repeats-containing protein termed HOS that mediates IκBα degradation by recognizing the phosphorylated IκBα and recruiting SCF-HOS-Roc1 E3 ligase (11, 12). The function of HOS and of a similar relative protein, β-TRCP, is required for NF-κB activation (reviewed in Ref. 13). In this study, we have investigated the role of HOS in the regulation of melanoma cell sensitivity to apoptosis and report for the first time that inhibition of HOS function can sensitize tumor cells to cytokine- or DNA damage-induced apoptosis.

Materials and Methods

Plasmids. To create a HOSΔF dominant negative expression vector, we amplified the cDNA encoding amino acids 180–597 of HOS with primers entailed with SalI and NotI recognition sequences. PCR products were digested with SalI and NotI restriction enzymes and subcloned as 5′-SalI-NotI-3′ fragments in pEVB vector (a gift of E. Spanopoulou). This vector is based on eukaryotic expression vector pEF-BOS (14) and provides the hemagglutinin tag at the NH2 terminus of the fusion proteins. The marker plasmid encoding GFP, pEGFP, was purchased from Clontech. The pCI-neo-based construct encoding stable mutant β-catenin1313V (15) was kindly provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD).

Cells and Transfections. Human melanoma Lu1205 cells (kindly provided by M. Herlyn, Wistar Institute, Philadelphia, PA) were maintained in MCDB153/L15 medium (4:1) supplemented with 5% fetal bovine serum, l-glutamine, and antibiotics at 37°C with 5% CO2. Transfections were performed with LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer’s recommendations. The amount of plasmids in transfection mixtures was kept constant by the addition of pCDNA3. Treatment with human recombinant TNF-α (20 ng/ml; R&D Systems), cycloheximide (10 μg/ml; Sigma), cisplatin (1 mM; Sigma), UV radiation (60 J/m2), or ionizing radiation (2 Gy) was performed 30 h after transfection.

DNA Fragmentation Analysis. Cells were harvested 16 h after treatment, fixed with 70% ethanol, washed with PBS, and resuspended in 0.5 mL of PBS X-100 containing propidium iodide (40 μg/ml) and DNase-free RNase A (1 mg/ml). Cells were incubated at 25°C for 30 min and analyzed on a FACS-Calibur flow cytometer (Beckton Dickinson) for propidium iodide and GFP fluorescence. The percentage of cells to the left of the diploid G0/G1 peak, which is diagnostic of hypodiploid cells that have lost DNA, was taken as the percentage of apoptotic cells.

Fluorescence Microscopy and Imaging Analysis for Apoptosis. Lu1205 cells were grown on glass coverslips (Fisher Scientific) placed in 35-mm dishes. After treatment with drugs or irradiations, medium was removed, and cells were processed as described previously (16). Briefly, cells were washed with PBS before fixation with 3.7% paraformaldehyde for 10 min. After rehydration in PBS, cells were stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole nuclear dye (Sigma) for 30 min in the dark. Coverslips were then washed with PBS, blotted dry, and mounted onto glass slides using Prolong Antifade mounting medium (Molecular Probes, Inc). Epifluorescence microscopy was performed using an Olympus Vanox AH-2 microscope (Olympus, Melville, NY) equipped with differential wavelength filters and a Zeiss Plan 40X/0.65 NA objective. Images were captured using an integrating Toshiba 3 chip color charge-coupled device camera (Toshiba, New York, NY) with a RGB color framegrabber (Flashpoint, I-Cube, Crofton, MD) and analyzed.
Results and Discussion

The HOS protein contains two important domains that are required for its function as a receptor for SCF-Roc1 E3 ubiquitin ligase (11). Seven WD40 repeats serve to recognize the DS(PO3)GXS(PO3)m motif on phosphorylated substrates and mediate HOS binding to those substrates (11). The F-box located at the NH2 terminus of HOS is a docking site for association with Skp1-Cullin1-Roc1 ubiquitin ligase complex (11, 12). Forced expression of HOS that lacks the F-box is impaired in the melanoma cells transfected with the dominant negative HOS construct. These data suggest that ubiquitination and degradation of IκBα in human melanoma cells is regulated by HOS/β-TRCP.

Stabilization of IκBα by HOS5F is expected to result in the sequestration of NF-κB in the cytoplasm and to down-regulate NF-κB transcriptional activity. Another effect of disruption of SCF-HOS-Roc1 function is stabilization of β-catenin and activation of β-catenin/Tcf-dependent transcription (11). Recent evidence that up-regulation of the β-catenin/Tcf pathway leads to the accumulation of an important regulator of apoptosis, p53 (17), prompted us to assess the level of p53 in Lu1205 cells. Lu1205 contains a detectable level of p53 (Fig. 1B, Lane 1) that is moderately increased in cells transfected with HOS5F (Fig. 1B, Lane 2). Expression of stable β-cateninS33Y mutant, which is not sensitive to HOS-mediated ubiquitination and degradation (15), led to a considerable accumulation of p53 (Fig. 1B, Lane 3).

To test whether HOS5F-mediated stabilization of IκBα or/and accumulation of p53 via stabilized β-catenin may affect the rate of apoptosis in Lu1205 cells, we used fluorescence-activated cell sorting-based DNA fragmentation assays. Because the overall transfection efficiency was about 20%, we cotransfected cDNA encoding GFP, the expression of which allows the analysis of cells that incorporated a plasmid (GFP-positive cells). Treatment of Lu1205 cells with TNF-α and cycloheximide led to a moderate increase in the rate of apoptosis of the overall cell population (Fig. 2). Transfection of Lu1205 with cDNAs encoding GFP alone or in combination with stable β-cateninS33Y mutant did not promote TNF-α/cycloheximide-induced cell death in the total cell population. Moreover, those transfections slightly inhibited apoptosis in the GFP-positive cells (Fig. 2). Conversely, transfection of HOS5F led to a 6-fold increase in the rate of apoptosis in GFP-positive cells (Fig. 2). These data indicate that inhibition of HOS function results in sensitization of Lu1205 melanoma cells to the programmed cell death induced by TNF-α with cycloheximide.

It is likely that the proapoptotic effect of HOS5F relies on inhibition of NF-κB activity because the expression of HOS5F results in stabilization of IκBα (Ref. 11; Fig. 1A). Overexpression of stable IκBα (deleted in its NH2 terminus) which is known to inhibit NF-κB (18) also sensitizes Lu1205 cells to TNF-α-induced cell death.4 It has been shown that expression of a stable IκBα mutant renders hepatocytes sensitive to TNF-α-induced apoptosis (19) and that adenovirus-mediated expression of dominant mutant IκBα promotes the cytotoxicity of TNF-α in human melanoma cells (8). The result that expression of stable β-cateninS33S mutant did not promote apoptosis (Fig. 2) argues against the suggestion that the effect of dominant negative HOS mutant is mediated by stabilization of β-catenin and the concurrent accumulation of p53 (Fig. 1B).

To confirm that inhibition of HOS function increases the susceptibility of human melanoma cells to apoptosis, we cotransfected Lu1205 cells with HOS5F and GFP constructs and treated them with TNF-α/cycloheximide as well as ionizing or UV radiation or the anticancer drug cisplatin. Apoptotic cells exhibiting the characteristic morphology (pyknosis, membrane blebbing, and cell body condensation; Fig. 3A) were scored among GFP-negative cells (which did not uptake GFP- and HOS-expressing plasmids) and GFP-positive cells. Expression of the dominant negative mutant of HOS resulted in an

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4 V. Ivanov and Z. Ronai, unpublished observations.
increase in the rate of apoptosis after treatment with TNF-α and cycloheximide (Fig. 3), thus confirming our DNA fragmentation analysis data (Fig. 2). Moreover, transfected cells exhibited a considerably higher level of programmed cell death in response to treatment with radiation or cisplatin (Fig. 3). Due to the loss of dying/dead cells during cell culture and sample processing (washes), the ratio of apoptosis in transfected/untransfected cells is probably underestimated. Nevertheless, these data suggest that inhibition of HOS function results in sensitization of human melanoma cells to apoptosis induced by DNA-damaging agents.

It has been suggested that NF-κB inhibiting agents could become useful adjuvants in antitumor therapies (9, 10). Inhibition of ubiquitination and degradation of IκBα by blocking the function of HOS/β-TRCP readily increased the killing of human melanoma cells by various cytotoxic agents. It is noteworthy that despite the apoptotic nature of cell death, this effect does not seem to depend on p53 function in Lu1205 cells, because p53 accumulation in response to overexpression of HOSΔF or β-cateninS33Y does not correlate with the rate of apoptosis (Figs. 1B and 2). The finding that inhibition of HOS function may promote apoptosis in melanoma cells is of importance because previous reports indicated that chemotherapy-induced apoptosis in melanoma cells is p53 dependent, and mutation of the p53 gene may lead to drug resistance (20). There is a possibility that HOS inhibition may increase anticancer drug-induced cell killing in tumor cells other than melanoma in a p53-independent manner.

At present, we cannot rule out that mechanisms other than down-regulation of NF-κB activity may participate in the HOS-dependent sensitization of melanoma cells to apoptosis. Identifying the mechanisms of HOS function and regulation may lead to the design of agents capable of inhibiting HOS function that may serve as a potential target for putative antitumor agents.

Acknowledgments

We thank M. Herlyn for Lu1205 melanoma cells and Bert Vogelstein for pCI-neo-β-cateninS33Y plasmid. Fluorescent microscopy was performed using the Microscopy/Imaging Shared Resources of the Lombardi Cancer Center, Georgetown University, Washington, D.C. (USPHS Grant 2P30-CA-51008).

References


Fig. 3. A, apoptotic morphology of Lu1205 cells transfected with pEGFP (0.2 μg) and HOSΔF (0.8 μg). Thirty h after transfection, cells were treated as indicated for 16 h, fixed, and stained with 4′,6-diamidino-2-phenylindole, and the apoptotic cells were scored among GFP-negative and GFP-positive cells. UT, untreated control. B, the percentage of the apoptotic cells shown in A was calculated (minus the values for untreated control) and depicted.
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