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Ubiquitin proteasomal pathway mediated degradation of p53 in melanoma

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ABSTRACT

Ubiquitin proteasomal pathway (UPP) is the principle mechanism for protein catabolism and affects cellular processes critical for survival and proliferation. Levels of tumor suppressor protein p53 are very low in cells due to its rapid turnover by UPP-mediated degradation. While p53 is mutated in human cancers, most human melanomas maintain wild-type conformation. In this study, to investigate the effects of UPP inhibitor *in vitro* and *in vivo,* we used a genetically-engineered mouse model (GEMM) that has the same genetic alterations as those of human melanomas. Melanoma cells were established from mouse tumors and named 8B20 cells. Treatment of 8B20 cells with the UPP inhibitors, MG132 and clasto-lactacystin-β-lactone, led to an increase in levels of p53 while treatment with non-proteasomal inhibitors did not alter p53 levels. UPP inhibitors induced formation of heavy molecular weight ubiquitinated proteins, a hallmark of UPP inhibition, and p53-specific poly-ubiquitinated products in 8B20 cells. To further decipher the mechanism of p53 stabilization, we investigated half-life of p53 in cells treated with cycloheximide to block *de novo* protein synthesis. Treatment of 8B20 cells with MG132 led to an increase in the half-life of p53. Further analysis revealed that p53 stabilization was not mediated by phosphorylation of Ser-15 and Ser-20 residues. *In vivo* studies showed that MG132 induced p53 overexpression and reduced tumor growth, suggesting an important role of p53 stabilization in controlling melanoma. Taken together, our studies provide a proof of principle for using a GEMM to address the mechanisms of action and efficacy of melanoma treatment.

Introduction

Several studies have shown that repeated exposures of UV irradiation to human skin results in both melanoma and non-melanoma skin cancers [1–4]. The tumor suppressor protein p53 is extensively investigated because of its role as a major tumor suppressor in humans and other mammals [5] (for a review see Ref. [6]). Loss or mutation of p53 is strongly associated with an increased susceptibility to cancer or neoplasia [7]. When normal cells are subjected to stress signals, such as DNA damage or oxidative stress, p53 is activated, resulting in transcription of downstream genes that coordinate either growth arrest or apoptosis of cells [8] to prevent proliferation and clonal expansion of damaged cells. Therefore, controlling p53-dependent pathway is a critical step in tumor initiation and progression. The loss of p53 activity is a major mechanism through which tumors become resistant to cell death, escape the local microenvironment and become recalcitrant to various types of therapeutic interventions.

Majority of cancers harbor point mutations in p53 which are likely in the central region of the protein responsible for DNA binding [9]. However, unlike other solid tumors, melanomas typically lack p53 mutations and retain the expression of wild-type p53 [10–13]. This is a surprising phenomenon given the highly malignant nature of the melanoma and its resistance to therapeutic interventions [14]. Melanoma development has been strongly associated with the deletions or mutations in the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) [15,16], leading to inactivation of $p16^{INK4a}$/cyclin-dependent kinases (CDK) 4 and 6/retinoblastoma protein ($p16^{INK4a}$/CDK4, 6/pRb) and $p14^{ARF}$/human double minute 2/p53 ($p14^{ARF}$/HDM2/p53) tumor suppressor pathways. The identification of small molecules and proteins that increase p53 stability and thereby protect cells against cancer progression is an active area of research [17].

In the current study, we explored whether the tumors with wild-type p53 such as melanoma, may benefit from the treatment strategies that activate p53. Here we utilize, for the first time, a genetically-engineered mouse model (GEMM) of melanoma with melanocyte specific mutant ras, deletion of *CDKN2A* and wild-type p53 that have the same genetic alterations as those of human

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melanomas to study the effects of proteasomal inhibitor MG132[2] on the melanoma both *in vitro* and *in vivo*.

Experimental

Chemicals and reagents

Cysteine protease inhibitor E-64, serine protease inhibitor PMSF, cycloheximide and bovine serum albumin were purchased from Sigma–Aldrich (St. Louis, MO). Protein A/G-agarose and clasto-lactacystin-β-lactone were obtained from Calbiochem/EMD Chemicals, Inc. (San Diego, CA). MG132 was purchased from BIOMOL International, L.P. (Plymouth Meeting, PA). Protease inhibitor cocktail tablets (cat #11 836 153 001) were obtained from Roche Diagnostics (Mannhein, Germany). All other chemicals and reagents used in this study were of highest purity grade available commercially.

Antibodies

Anti-ubiquitin (rabbit polyclonal) and anti-actin (mouse monoclonal) antibodies were purchased from Sigma–Aldrich. Anti-p53 (rabbit polyclonal) antibody was obtained from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK). Phospho-p53 (Ser-15) and phospho-p53 (Ser-20) antibodies were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA).

Animals

Animals used in the current study were Tyr-H-ras^{G12V} transgenic mice (FVB/N background) and Ink4a/Arf knockout (FVB/N background) obtained from National Cancer Institute at Frederick (Frederick, MD). These animals were cross-bred twice to generate transgenic mice with melanocyte-specific H-ras^{G12V} expression on an Ink4a/Arf-deficient background. Animals were housed at UCD vivarium under specific pathogen-free conditions according to the National Institutes of Health Animal Care Guidelines under an Institutional protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). These transgenic mice spontaneously develop cutaneous melanoma with high penetrance (74%) at around 3 to 4 months of age. The melanomas are amelanotic but express melanocyte pigment genes such as MART-1 and TRP-2.

Cell lines

8B20 mouse melanoma cells were established from a melanoma tumor spontaneously arising in the transgenic mice with melanocyte-specific H-ras^{G12V} expression on an Ink4a/Arf-deficient background. B16F10, a murine melanoma cell line, was obtained from the American Type Culture Collection (ATCC; Manassas, VA).

SDS–PAGE, immunoprecipitation and immunoblotting analysis

Exponentially growing cells were seeded at $\sim 2 \times 10^6$ in 100 mm plates, and 12 h later the cells were harvested. For immunoprecipitation studies, medium was removed and cells were washed with phosphate-buffered saline (PBS, pH 7.4), lysed on ice in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100 and 1 mM PMSF (pH 7.4) with a freshly added protease inhibitor cocktail (Roche Diagnostics), and then centrifuged at 13,200*g* for 10 min. Ten micrograms of the appropriate antibody was added to the supernatants and the mixture was gently rotated at 4 °C for 12 h. Protein A/G-agarose (40 μL) was then added, and the incubations were continued for additional 90 min. The protein A/G-agarose was collected by centrifugation and the beads were washed three times with 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 1% (v/v) Nonidet P-40. Protein A/G-agarose beads were then suspended in 2× Laemmli [18] SDS sample buffer and heated to 90 °C for 5 min. Immunoprecipitated proteins were analyzed by SDS–PAGE (12% gels) followed by transfer to a polyvinylidene difluoride membrane (0.4 μM) in 25 mM Tris, 192 mM glycine, and 20% methanol at 110 V for 1 h. Membranes were blocked overnight in 10 mM Tris–HCl, pH 8.0, 125 mM NaCl and 0.2% (v/v) Tween 20 containing 5% (w/v) nonfat dry milk (TBST-M5).

Immunoblot analysis of the total poly-ubiquitinated proteins and p53 specific poly-ubiquitinated forms were analyzed using anti-ubiquitin antibodies. For the determination of cellular levels of proteins, cells were scraped after the respective treatments, washed with PBS, lysed in RIPA buffer and then sonicated on ice. The supernatant was obtained by centrifugation at 13,200*g* for 5 min, and the protein concentration was determined by the method of Lowry [19]. Protein bands were visualized using enhanced chemiluminescence as described by the manufacturer (Pierce Biotechnology, Inc., Rockford, IL). Densitometric analysis of the membranes was performed using GelDoc 200 (Bio-Rad Laboratories, Hercules, CA).

Inhibitor studies

Cells were grown to 85% confluency in 60 mm dishes in complete minimal essential medium. Fresh medium containing various inhibitors MG132, clasto-lactacystin-β-lactone, E-64 and PMSF were added to each plate and the medium was replaced with fresh dose of the inhibitor 3 h later. After 6 h of incubation with various inhibitors, cells were washed in PBS and then lysed in ice-cold RIPA buffer. Protein concentrations were determined and target proteins were analyzed by immunoblotting.

p53 Protein turnover studies

p53 protein stability was determined in the presence or absence of proteasomal inhibitor MG132 using 8B20 cells. Approximately 2×10^7 cells were plated into 100 mm plates, and 12 h later fresh medium containing cycloheximide (50 μg/mL) was added. At indicated time intervals, cells were scraped and pelleted by centrifugation at 13,200*g* for 10 min. Cells were lysed in RIPA buffer and supernatant collected as described above. To assess the p53 turnover in the presence of proteasomal inhibitor MG132, cells were treated with MG132 (10 μM) and cycloheximide at a concentration of 50 μg/mL simultaneously to block the *de novo* protein synthesis. Proteasomal inhibitor and cycloheximide were present in the medium during the entire duration of the experiment.

Cell cycle analysis

Cells were synchronized by growth confluence, harvested by trypsinization and re-plated in 100 mm plates. After 24 h cells were treated with varying concentrations of the proteasomal inhibitor MG132 (0–10 μM) for 24 h. Cells were then washed with PBS and an aliquot of cells was stained with Krishan's stain [20] at 4 °C for

[2] *Abbreviations used:* UPP, ubiquitin proteasomal pathway; MG132, Z-Leu-Leu-Leu-CHO; LC, clasto-lactacystin-β-lactone; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation; SDS-PAGE; sodium dodecyl sulphate polyacrylamide gel electrophoresis.