Insulin-like Growth Factor I Induces MDM2-dependent Degradation of p53 via the p38 MAPK Pathway in Response to DNA Damage

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In many tissues, the insulin-like growth factor I (IGF-I) receptor (IGF-IR) is known to functionally oppose apoptosis. Recently, we demonstrated a direct role for the IGF-IR in the rescue of DNA-damaged fibroblasts by activating a DNA repair pathway (Héron-Milhavet, L., Karas, M., Goldsmith, C. M., Baum, B. J., and LeRoith, D. (2001) J. Biol. Chem. 276, 18185–18192). p53 is a nuclear transcription factor that can block progression of the cell cycle, modulate DNA repair, and trigger apoptosis. In this work, we tested the effect of IGF-I on the regulation of the p53 signaling cascade. The DNA-damaging agent 4-nitroquinoline 1-oxide was applied to NIH-3T3 cells overexpressing normal IGF-IRs (NWTb3 cells). We showed that after 4-nitroquinoline 1-oxide-induced DNA damage, IGF-I induced exclusion of the p53 protein from the nucleus and led to its degradation in the cytoplasm, whereas p53 mRNA was unaffected. Degradation of the p53 protein was associated with an increase in MDM2, an upstream modulator of the half-life and activity of the p53 protein. p53 degradation was also associated with down-regulation of p21. We further showed that the effects of IGF-I on mdm2 transcription and on MDM2/p19 ARF association were mediated by the p38 MAPK pathway. In conclusion, we describe a novel role for IGF-I in the regulation of the MDM2/p53/p21 signaling pathway during DNA damage.

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a membrane-bound heterotetramer with ligand-induced tyrosine kinase activity (1). The IGF-IR is expressed by many tumors and, upon activation by IGFs, plays an important role in cell growth by inducing mitogenesis and transformation and by protecting cells from apoptosis (2). IGF-I can protect fibroblasts from apoptosis induced by UVB light via activation of the Akt protein kinase signaling pathway (3). It has also recently been demonstrated that activation of the IGF-IR can rescue 4-nitroquinoline 1-oxide (4-NQO)-damaged cells by activating a DNA repair pathway predominantly mediated by signaling through the stress-activated protein kinase p38 (4).

p53 is a tumor suppressor gene that is one of the most commonly mutated genes in human cancers. The role of p53 in tumor suppression was demonstrated in p53−/− (knockout) mice, which are highly prone to developing a variety of different cancers (5). The specific biological roles attributed to p53 are very complex, but p53 essentially protects the genome from mutations and genetic alterations. Structural analyses have shown that p53 is a transcription factor with a sequence-specific DNA-binding domain in its central region and a transcription activation domain at its N terminus (6). Under basal conditions, cellular p53 protein levels are relatively low. However, in response to DNA damage and/or various stresses, the p53 protein is stabilized, and its ability to bind specific DNA sequences is activated. Both stabilization and DNA binding are modulated by post-translational modifications of the p53 protein, including phosphorylation and acetylation (7, 8). The upstream regulatory interactions and downstream events involved in stabilization of p53 are part of a complex network of signaling pathways that lead to either cell cycle arrest or apoptosis (6). A number of genes have been identified as primary targets of p53 (6, 9). In response to DNA damage, stabilization of p53 blocks the cell cycle by binding directly to genomic p53 response elements and stimulating the expression of p21waf1/cip1, an inhibitor of cyclin-dependent kinases (10). It has been reported that p21waf1/cip1 is mainly responsible for p53-induced G1 arrest (11). GADD45 is another target of p53 and may participate in the coupling between chromatin assembly and DNA repair (9). Zhao et al. (12) demonstrated that these two p53 targets, p21 and GADD45, act upon different signaling pathways that suppress cell growth.

One crucial regulator of p53 is the cellular protein MDM2 (13). MDM2 can both inhibit the function of p53 and target p53 for degradation in the cytoplasm (see Ref. 14 for review). The MDM2 protein is a RING finger ubiquitin ligase that can induce destabilization and subsequent ubiquitin-mediated proteasomal degradation of p53 (15).

Previous studies have established a relationship between IGF-I, cell growth, and p53. For example, it has been shown that the anti-apoptotic effect of IGF-I in myocytes is mediated by its ability to depress p53 transcriptional activity (16). Moreover, IGF-I-induced growth stimulation in MCF-7 cells is accompanied by tyrosine phosphorylation and nuclear exclusion of p53 (17). Recently, we demonstrated a role for IGF-IR activation in DNA repair in response to 4-NQO-induced damage in NIH-3T3 fibroblast cells (4). Thus, it was of particular interest to determine whether, in our experimental system, stimulation of the IGF-IR exerts its effects through the modulation of p53 and its network of transcriptional events and effects.

EXPERIMENTAL PROCEDURES

Materials—4-NQO was purchased from Sigma, and recombinant human IGF-I was a gift of Genentech (San Francisco, CA). Anti-p53 (Pab240) and anti-p21 (M19) antibodies used for Western blotting were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse anti-MDM2 antibody was a gift from Dr. G. Lozano (M. D. Anderson Cancer Institute, Houston, TX), and the mouse anti-p124Ab

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1 The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor I receptor; 4-NQO, 4-nitroquinoline 1-oxide; ARF, p19 alternate reading frame; HA, hemagglutinin; E3, ubiquitin-protein isopeptide ligase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.
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antibody was obtained from Novus Biologicals (Littletone, CO). Horse-radish peroxidase-conjugated secondary antibodies, including antimouse and anti-rabbit IgG, were obtained from Amersham Biosciences. The anti-HA antibody was obtained from Covance (Vienna, VA), and the fluorescein isothiocyanate-conjugated secondary antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Specific protein kinase inhibitors (LY294002, SB202190, and U0126) were from Calbiochem. The mouse RNA probe templates for mdm2, p53, and 18 S RNA used for Northern blots were purchased from Ambion Inc. (Austin, TX). The RNA probe templates for p19ARF were kindly provided by Drs. T. Ouchi (Mt. Sinai Medical School, New York, NY) and C. Sherr (St. Jude Children’s Research Hospital, Memphis, TN).

Cell Culture—The NWTb3 cell line established in this laboratory expresses wild-type human IGF-1R at a level of ~4 × 10^5 receptors/cell (18). This cell line was routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 300 µg/ml l-glutamine, and 0.5 µl/liter G418 in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

Transient Transfection—NWTb3 cells were seeded the day prior to transfection. The cells were then transiently transfected using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. For immunofluorescence, cells were transfected with the pcDNA3-HAp53 construct (human p53), kindly provided by Dr. W. G. Kaelin, Jr. (Dana Farber Cancer Research Institute, Boston, MA). After transfection, the cells were subjected to immunofluorescence microscopy and immunoblotting to assess the efficiency of the transient transfection. The transfection efficiency in NWTb3 cells was determined in our previous study using FuGENE 6 transfection reagent (4).

NWTb3 cells were also transfected with a dominant-negative p38 construct (pCMV5-p38AGF) with the FuGENE 6 transfection reagent as previously described (4). The cells were then treated with 4-NQO in the absence or presence of IGF-I. Total RNA was extracted, and mdm2 Northern blotting was performed as described below.

4-NQO and UV Light Cell Treatment, Immunoprecipitation, and Immunoblotting—NWTb3 cells were cultured in 100-mm dishes (1 × 10^6 cells/dish) as described above. One day after plating, the cells were starved overnight in serum-free medium. The UV-mimetic drug 4-NQO was dissolved in 100% ethanol at a concentration of 2 mM. A 100 µM stock solution was prepared by diluting the concentrated solution in serum-free medium and stored in aliquots at −20 °C for use throughout the study. All 4-NQO exposures were performed at a final concentration of 2.5 µM in serum-free medium for 30 min at 37 °C in a humidified CO₂ incubator. Following the drug treatment, cells were rinsed twice with prewarmed (37 °C) serum-free medium. The cells were then incubated for 1 h at 37 °C in the absence or presence of specific protein kinase inhibitors at the appropriate concentrations. Two different doses of UV treatment were used: a low dose of 6 J/m² and a high dose of 24 J/m² (19), known to increase p53 protein levels (20). The cells were rinsed twice with serum-free medium, and both 4-NQO- and UV-treated cells were then incubated in the presence or absence of IGF-I for various times and at various concentrations as indicated on the figures. The cells were then washed with ice-cold phosphate-buffered saline, and total cell lysates were prepared exactly as described previously (4). For immunoprecipitation experiments, aliquots of lysates containing 400 µg of protein were incubated with 40 µl of the anti-p19ARF antibody (0.1 µg/µl) at 4 °C overnight, and protein G-Sepharose beads were added to the lysate. Supernatants were subjected to SDS-PAGE on a 4–20% gradient gel and immunoblotting with the anti-MDM2 antibody. All antibodies used (anti-p53, anti-actin, and anti-p21) were diluted in Tris-buffered saline and 0.1% Tween 20 supplemented with 5% bovine serum albumin according to the manufacturers’ recommended conditions, except for the anti-ARF antibody, which was diluted in Tris-buffered saline and 0.1% Tween 20, and the anti-MDM2 antibody, which was diluted in 0.1% Tween 20 supplemented with 5% nonfat dry milk.

Immunofluorescence Microscopy—Immunofluorescence was performed on NWTb3 cells transiently transfected with HA-tagged human p53 as described above. Briefly, cells were seeded on Nunc chamber slides and then washed and fixed in 2% formaldehyde solution. The samples were blocked for 15 min with Dulbecco’s PBS and 10% fetal bovine serum and incubated with the mouse anti-HA primary antibody for 1 h at room temperature in Dulbecco’s PBS and 10% fetal bovine serum. Samples were then incubated with the fluorescein isothiocyanate-conjugated anti-mouse secondary antibody for 30 min at room temperature in the same buffer. Slides were mounted with mounting medium and sealed. Samples were viewed using an inverted fluorescence microscope equipped with a fluorescein isothiocyanate filter (Axiovert 100S, Zeiss) at a magnification of ×64.

Northern Blotting—Cells were homogenized in Trizol reagent (Invitrogen), and total RNA was isolated as recommended by the manufacturer. Total RNA (20 µg) was loaded onto a 1.25% agarose gel containing formaldehyde. Ethidium bromide staining was utilized to assess the integrity of the RNA and to estimate loading efficiencies. After electrophoresis, RNA was transferred onto Nytran nylon membrane (Schleicher & Schuell) by capillary action and fixed by UV cross-linking. mRNA levels were determined by hybridizing Northern blots with [32P]-labeled riboprobes specific for mdm2, p53, and 18 S mRNA or with DNA probes for p19ARF and excision repair cross-complementing 1 (ercc-1). Quantification of the specific message according to the 1.8-kb 18 S message was performed using Image Reader software and Image Gauge software together with a Fuji 1800II instrument.
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RESULTS

IGF-I Induces a Time- and Dose-dependent Decrease in p53 Protein Levels in Response to DNA Damage—Our study was performed in NWTb3 cells overexpressing normal human IGF-IRs (18). Recent studies in our laboratory have shown that the IGF-IR plays a role in rescuing these cells from DNA damage (4). Given the importance of the tumor suppressor gene p53 in the regulation of apoptosis and because IGF-I signaling plays a role in DNA repair, we examined the effect of IGF-IR activation on p53 protein levels in response to DNA damage. As described above, the cells were treated with 4-NQO (2.5 μM) and stimulation with a physiological concentration of IGF-I (50 nM). p53 protein levels were determined by Western blotting samples treated with 4-NQO alone, IGF-I alone, or 4-NQO followed by IGF-I. No significant changes in p53 levels were observed in cells treated with 4-NQO alone (Fig. 1A). Both the time course (0–6 h) and the dose dependence (0–100 nM) of IGF-I effects were evaluated (Fig. 1, A and B, respectively). After induction of DNA damage with 4-NQO, IGF-I treatment resulted in a 50% decrease in the level of p53 after 6 h of stimulation (Fig. 1A). A slight decrease in p53 protein levels was observed when the cells were stimulated with IGF-I alone for 6 h, but this effect did not exhibit dose dependence (Fig. 1B). On the other hand, a marked dose-dependent reduction in the expression of p53 was observed in samples that were treated with IGF-I for 6 h after 4-NQO exposure (Fig. 1B). These data suggest that IGF-IR activation plays a role in the regulation of p53 protein levels in NWTb3 cells and that this effect is most pronounced after cellular DNA damage has already occurred.

IGF-I Induces Intracellular Localization Shuttling of p53 from the Nucleus to the Cytoplasm—To determine the cellular localization of p53, NWTb3 cells were transiently transfected with HA-tagged human p53. Western blotting with a specific antibody against human p53 confirmed that the human p53 protein was expressed (data not shown). Using a fluorescein isothiocyanate-conjugated antibody against the HA epitope tag, immunofluorescence microscopy was used to localize p53 under various conditions (4-NQO only, IGF-I only, or 4-NQO + IGF-I). In a control experiment, cells were transfected with p53, but did not receive any treatment. As shown in Fig. 2A, p53 was present in the nucleus and more specifically in the nucleoplasm under these conditions. No fluorescence was detected in the non-transfected NWTb3 cells (data not shown). When cells were treated with IGF-I for 2 h (Fig. 2B) or for 5 h (Fig. 2C), no differences were observed in p53 localization. Similarly, no differences in the cellular localization of p53 were observed in cells exposed to 4-NQO alone for 2 or 5 h (Fig. 2, D and E, respectively). In striking contrast, when 4-NQO-treated cells were subsequently exposed to IGF-I, p53 localization was partially shifted from the nucleoplasm to the cytoplasm of NWTb3 cells in a time-dependent manner (Fig. 2, F and G). Thus, in response to 4-NQO-induced DNA damage, stimulation with IGF-I promotes the shuttling of p53 from the nucleus to the cytoplasm.

IGF-I Effect on mdm2 and p53 mRNA Levels—MDM2 is a proto-oncoprotein that is known to negatively regulate p53 (21). Thus, we also studied the effects of 4-NQO and IGF-I on MDM2 and investigated whether MDM2 plays a role in the reduction of p53 protein levels. First, we focused on the decrease observed in p53 protein levels after DNA damage and subsequent IGF-I treatment. Northern blot analysis was used to assess mdm2 and p53 mRNA levels. As shown in Fig. 3A, p53 mRNA levels stayed relatively constant after IGF-I, 4-NQO, or 4-NQO + IGF-I treatment, suggesting that the decrease in p53 protein levels shown in Fig. 1 was due to a post-translational effect. A 2-h incubation with IGF-I increased mdm2 mRNA levels by ~2-fold, whereas 4-NQO exposure followed by IGF-I treatment increased mdm2 mRNA levels by ~4-fold. This effect appeared to plateau at the 2-h time point. These results were confirmed by measuring mdm2 mRNA levels in an RNase protection assay (data not shown). Moreover, the MDM2 protein was also slightly increased in response to 4-NQO exposure followed by IGF-I treatment, although to a lesser degree than the mRNA (~2-fold), as shown in Fig. 3B. Because MDM2 is a negative regulator of the p53 protein, the results presented may suggest that IGF-I stimulation occurring after DNA damage plays a role in the degradation of the p53 protein via induction of MDM2.

IGF-I Decreases p53 Target Gene Expression—To determine the functional consequences of changes in p53 protein levels, we studied the expression of p21WAF1, a downstream target of p53 (21). Thus, we also studied the effects of 4-NQO and IGF-I on MDM2 and investigated whether MDM2 plays a role in the reduction of p53 protein levels. First, we focused on the decrease observed in p53 protein levels after DNA damage and subsequent IGF-I treatment. Northern blot analysis was used to assess mdm2 and p53 mRNA levels. As shown in Fig. 3A, p53 mRNA levels stayed relatively constant after IGF-I, 4-NQO, or 4-NQO + IGF-I treatment, suggesting that the decrease in p53 protein levels shown in Fig. 1 was due to a post-translational effect. A 2-h incubation with IGF-I increased mdm2 mRNA levels by ~2-fold, whereas 4-NQO exposure followed by IGF-I treatment increased mdm2 mRNA levels by ~4-fold. This effect appeared to plateau at the 2-h time point. These results were confirmed by measuring mdm2 mRNA levels in an RNase protection assay (data not shown). Moreover, the MDM2 protein was also slightly increased in response to 4-NQO exposure followed by IGF-I treatment, although to a lesser degree than the mRNA (~2-fold), as shown in Fig. 3B. Because MDM2 is a negative regulator of the p53 protein, the results presented may suggest that IGF-I stimulation occurring after DNA damage plays a role in the degradation of the p53 protein via induction of MDM2.

Regulation of MDM2-dependent p53 Degradation—To further characterize MDM2-induced p53 degradation, we investigated the effects of DNA damage and subsequent IGF-I treatment on p19ARF, which is known to inhibit MDM2 and to indirectly stabilize p53. Western blot analysis was used to...
determine p19ARF protein levels in response to the various treatments. As shown in Fig. 5, p19ARF protein levels did not vary significantly in response to exposure to 4-NQO, IGF-I, or 4-NQO + IGF-I. p19ARF mRNA levels were also determined by Northern blot analysis. p19ARF mRNA levels are relatively low in NIH-3T3 cells (23). Nevertheless, no changes were observed in p19ARF mRNA levels (data not shown), in agreement with the lack of effect of the p19ARF protein. These results suggest that p19ARF may not be directly involved as an upstream component in the MDM2/p53 signaling pathway targeted by IGF-I in response to DNA damage.

IGF-I Prevents Association of MDM2 with ARF after 4-NQO Treatment—One factor that regulates p53 stabilization involves the interaction between p19ARF and MDM2, which inhibits MDM2 ubiquitin-protein isopeptide ligase (E3) activity and consequently stabilizes p53. Thus, we studied the effect of IGF-I on the association between the two proteins. Fig. 6 shows a representative experiment in which ARF was immunoprecipitated from NWTb3 cell lysates, and the resulting samples were immunoblotted with the anti-MDM2 antibody. In the absence of DNA damage, the association between ARF and MDM2, which is known to stabilize the p53 protein, reached a maximum 2 h after IGF-I treatment (in the absence of 4-NQO-induced DNA damage), as shown in Fig. 6. To investigate whether the effect seen with 4-NQO and IGF-I may be 4-NQO-specific, we used UV light (254 nm) to irradiate NWTb3 cells, and we treated the cells with IGF-I (50 nM) for 0, 2, 6, and 24 h. Two different doses of UV light were used: a low and a high dose of UV radiation, previously described to increase p53 protein levels (24). Fig. 7 shows a typical experiment in which p19ARF was immunoprecipitated from NWTb3 cell lysates. Immunoblotting was then performed with the anti-MDM2 antibody. In the absence of DNA damage, the association between ARF and MDM2, which is known to stabilize the p53 protein, reached a maximum 2 h after IGF-I treatment (in the absence of 4-NQO-induced DNA damage), as shown in Fig. 6. To investigate whether the effect seen with 4-NQO and IGF-I may be 4-NQO-specific, we used UV light (254 nm) to irradiate NWTb3 cells, and we treated the cells with IGF-I (50 nM) for 0, 2, 6, and 24 h. Two different doses of UV light were used: a low and a high dose of UV radiation, previously described to increase p53 protein levels (24). Thus, in the presence of UV- or UV-mimetic-induced DNA damage, IGF-I treatment failed to promote the association between ARF and MDM2 and therefore would not be expected to suppress MDM2 E3 activity. Thus, these findings suggest that treatment with IGF-I after UV-induced DNA damage may promote degradation of the p53 protein, which is consistent with the data shown in Fig. 1.

p38 MAPK Is Implicated in the Regulation of MDM2 and MDM2/ARF Association by IGF-I after DNA Damage—In a previous study, we showed that NWTb3 cells can be rescued from 4-NQO-induced cell proliferation inhibition by activation of the IGF-IR and that this effect is mediated primarily...
through the p38 MAPK pathway (4). To determine whether the p38 MAPK pathway plays a role in the regulation of mdm2 gene expression, we treated the NWTb3 cells with 4-NQO and IGF-I in the presence or absence of compounds that inhibit various protein kinases. The increase in mdm2 mRNA induced by treatment with 4-NQO + IGF-I was completely blocked in cells pretreated with the p38 MAPK inhibitor SB202190, but not in cells pretreated with a phosphatidylinositol 3'-kinase inhibitor or an ERK1/2 MAPK inhibitor (data not shown). Moreover, we investigated whether p38 MAPK is involved in the regulation of MDM2/ARF association. When the cells were treated with SB202190 (after 4-NQO exposure and before IGF-I stimulation), the association between the two proteins could be restored, as shown in Fig. 8. This result indicates that in addition to a slight regulation of the MDM2 protein levels, p38 MAPK seems to be involved more specifically in the modulation of MDM2/ARF association to promote degradation of the p53 protein.

Fig. 5. Effects of 4-NQO and IGF-I on p19ARF protein levels. NWTb3 cells were incubated in the presence or absence of 4-NQO (2.5 μM) for 30 min, followed by vehicle or 50 nM IGF-I for various times as indicated. At each time point, total cell lysates were prepared and subjected to immunoblotting with the anti-p19ARF antibody. The membranes were then reblotted with the anti-actin antibody to assess equal protein loading. The immunoblot is representative of data obtained in two similar experiments. The lower panel shows quantitation of immunoreactivity levels.

Fig. 6. Effects of IGF-I and 4-NQO on the association between p19ARF and MDM2 proteins. NWTb3 cells were incubated in the presence or absence of 4-NQO (2.5 μM) for 30 min, followed by vehicle or 50 nM IGF-I for various times; and the cells were harvested at the indicated times after IGF-I treatment. Aliquots of cell lysates containing a total of 400 μg of protein were incubated with the anti-p19ARF antibody as described under “Experimental Procedures.” The immunoprecipitation samples were loaded onto a 4–20% SDS-polyacrylamide gel. Western blotting (WB) was performed using the anti-MDM2 antibody diluted in 5% nonfat dry milk. The membrane was reblotted with the anti-p19ARF antibody to determine whether equal amounts of p19ARF protein had been immunoprecipitated (IP). The data shown are representative of two similar experiments, although in one other experiment, the maximal interaction was observed after 3 h of IGF-I treatment.

Fig. 7. Effects of IGF-I and UV light on the association between MDM2 and p19ARF. NWTb3 cells were treated with UV light at two different doses: 6 J/m² (low dose) and 24 J/m² (high dose). The cells were then harvested at the indicated times after IGF-I treatment, and protein lysates were prepared. Immunoprecipitation (IP) was performed as described in the legend to Fig. 6. WB, Western blotting.

DISCUSSION

We have investigated the role of the IGF-IR in the regulation of the p53 network and its relationship to DNA repair in response to UV-mimetic (4-NQO)-induced DNA damage. Previously, IGF-I has been clearly implicated both in the protection of cells from apoptosis and in DNA repair. In this report, we now provide evidence that p53, MDM2, and p19ARF are part of the pathway used by IGF-I to promote DNA repair in damaged cells.

In the context of 4-NQO-induced DNA damage, subsequent IGF-I treatment induced an increase in mdm2 mRNA levels with concomitant decreases in p53 protein levels and p53 functional activity (as shown by reduced levels of the p21 protein). In UV-mimetic-treated cells, IGF-I failed to induce an association between p19ARF and MDM2. The lack of association between p19ARF and MDM2 (association that appears to be p38 MAPK-dependent) thereby enables activation of MDM2 and facilitates MDM2-induced degradation of p53. Moreover, IGF-I promoted the nuclear export of p53 in 4-NQO-treated cells, thus further promoting its degradation through MDM2 (a negative regulator of p53).

Three competing models have been described to explain the nuclear export of p53 (14). The first model proposes that MDM2 binds to p53 in the nucleus and shuttles p53 to the cytoplasm (25). The second model suggests that p53 itself contains a nuclear export signal capable of mediating its own nuclear export (26). Finally, the third model proposes that MDM2 ubiquitinates p53 in the nucleus and that this ubiquitination promotes its nuclear export and subsequent cytoplasmic degradation (27). Although all three models suggest that nuclear transport is necessary for the degradation of p53, none explain why p53 must transfer to the cytoplasm to be degraded by the proteasome. No regulation of ARF mRNA or protein could be detected when the cells were treated with IGF-I after 4-NQO-induced DNA damage. However, IGF-I was shown to regulate MDM2 activity by inhibiting the association between ARF and MDM2 in a p38 MAPK-dependent manner. ARF also plays an important role in stabilizing p53 by inhibiting MDM2-mediated p53 degradation, at least in part by blocking the nuclear export of p53 and MDM2. The function of mouse p19ARF (or human p14ARF) (23) may involve direct binding to MDM2, thereby leading to the stabilization of p53 (21). The MDM2/ARF interaction stabilizes p53 by a mechanism that involves inhibition of the intrinsic E3 activity of MDM2, which is required for p53 degradation (28). Inhibition of MDM2-induced shuttling of p53 from the nucleus to the cytoplasm, where it is degraded by the proteasome, may also represent a mechanism by which the MDM2/ARF interaction stabilizes p53 (29). ARF is found predominantly in the nucleolus, whereas MDM2 and p53 are usually nucleoplasmic and shuttle in and out of the nucleus to deliver ubiquitinated p53 to the cytoplasmic proteasome (29). It has been proposed that nucleolar localization is not essential for the function of ARF, but that it may enhance the availability of ARF to inhibit MDM2 (21).
Thus, when IGF-I was used to rescue NWTb3 cells from UV-mimetic-induced DNA damage, the p53 protein was degraded through the MDM2/ARF-mediated pathway. Our data are consistent with a secondary down-regulation of p21 in response to a decrease in p53 when cells are sequentially exposed to 4-NQO and then to IGF-I. This may occur as a mechanism of rescuing NWTb3 cells from DNA damage through the degradation of p53.

Nucleotide excision repair is considered to be the major repair system that cells use to eliminate a large range of DNA lesions. The identification of DNA lesions is a complex process whereby lesions are localized, a stretch of nucleotides containing the damage is excised, and a repair patch is synthesized (for a review on DNA repair, see Ref. 30). Nucleotide excision repair genes, including ERCC-1 and XBP/ERCC-3, are thought to determine cellular sensitivity to a variety of DNA lesions (31). The human ERCC-1 gene is a DNA repair gene (32). The ERCC-1 protein has been shown to play a crucial role in the early excision step of damaged DNA by virtue of its intrinsic structure-specific nuclease activity. In a previous study, it has been shown that overexpression of the IGF-IR induces an increase in ERCC-1 gene expression in Chinese hamster ovary cells (33). A correlation between ERCC-1 levels and protection from UV-induced cell death was also established (33). Supraphysiological concentrations of insulin have also been shown to increase ERCC-1 mRNA levels in serum-deprived cells overexpressing insulin receptors (34). We found that IGF-I induced a time-dependent 2-fold increase in ERCC-1 mRNA levels in the absence or presence of 4-NQO (data not shown). Furthermore, this IGF-I-induced increase in ERCC-1 gene expression was p38-dependent (data not shown).

We propose a model for the mode of action of IGF-I in response to UV-mimetic-induced DNA damage (Fig. 9). Under basal conditions, IGF-I facilitates the association of p19^{ARF} with MDM2. However, after UV-induced DNA damage, IGF-I treatment cannot promote this association. Thus, MDM2 remains active (i.e. not inhibited by p19^{ARF}) and can ubiquitinate p53, thereby targeting it to the cytoplasm for proteasomal degradation. As a consequence, there is a decrease in p53 activity, as evidenced by down-regulation of p21.

In summary, this study demonstrates that p38 MAPK mediates IGF-I regulation of both induction of DNA repair and modulation of the MDM2/ARF/p53 network. Thus, we have identified a signaling cascade involving p53 as a target for IGF-IR activation. This complements our previous work showing that IGF-I promotes DNA repair in damaged cells through p38 MAPK (4). This work may lead to new areas of research regarding the role of p38 MAPK in cell growth and protection pathways.

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