A Sensitive Flow Cytometry-based Nucleotide Excision Repair Assay Unexpectedly Reveals That Mitogen-activated Protein Kinase Signaling Does Not Regulate the Removal of UV-induced DNA Damage in Human Cells

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In response to diverse genotoxic stimuli (e.g. UV and cisplatin), the mitogen-activated protein kinases ERK1/2, JNK1/2, and p38α/β become rapidly phosphorylated and in turn activate multiple downstream effectors that modulate apoptosis and/or growth arrest. Furthermore, previous lines of evidence have strongly suggested that ERK1/2 and JNK1/2 participate in global-genomic nucleotide excision repair, a critical antineoplastic pathway that removes helix-distorting DNA adducts induced by a variety of mutagenic agents, including UV. To rigorously evaluate the potential role of mitogen-activated protein kinases in global-genomic nucleotide excision repair, various human cell strains (primary skin fibroblasts, primary lung fibroblasts, and HCT116 colon carcinoma cells) were treated with highly specific chemical inhibitors, which, following UV exposure, (i) abrogated the capacities of ERK1/2, JNK1/2, or p38α/β to phosphorylate specific downstream effectors and (ii) characteristically modulated cellular proliferation, clonogenic survival, and/or apoptosis. A highly sensitive flow cytometry-based nucleotide excision repair assay recently optimized and validated in our laboratory was then employed to directly demonstrate that the kinetics of UV DNA photoadduct repair are highly similar in mock-treated versus mitogen-activated protein kinase inhibitor-treated cells. These data on primary and tumor cells treated with pharmacological inhibitors were fully corroborated by repair studies using (i) short hairpin RNA-mediated knockdown of ERK1/2 or JNK1/2 in human U2OS osteosarcoma cells and (ii) expression of a dominant negative p38α mutant in human primary lung fibroblasts. Our results provide solid evidence for the first time, in disaccord with a burgeoning perception, that mitogen-activated protein kinase signaling does not influence the efficiency of human global-genomic nucleotide excision repair.

Nucleotide excision repair (NER) is the only pathway available to human cells for the removal of helix-distorting (replication- and transcription-blocking) “bulky” DNA adducts generated by a multitude of environmental carcinogens. Among these adducts is the highly promutagenic UV-induced cyclobutane pyrimidine dimer (CPD), which lies at the origin of sunlight-associated mutagenesis and skin cancer development (1). The physiological importance of NER is highlighted by xeroderma pigmentosum (XP), a rare genetic disorder characterized by defective removal of bulky DNA adducts, UV hypersensitivity, and striking predisposition to skin cancer (2). Furthermore, NER status of tumors in cancer patients has been identified as a major determinant in the clinical response to UV-mimetic chemotherapeutic agents, such as cisplatin, which exert antineoplastic effects via the induction of bulky DNA adducts (3).

NER is composed of two distinct subpathways (i.e. global genomic-NER (GG-NER) removes bulky adducts from the genome overall, whereas transcription-coupled NER (TC-NER) removes such adducts exclusively from the transcribed strands of active genes) (see Ref. 4 for an excellent overview). These subpathways differ only in the mechanism of lesion recognition. GG-NER is triggered when the UV-DDB1/UV-DDB2 heterodimer recognizes and binds the helical distortion created by bulky adducts, which is followed by recruitment of the XPC-hHR23B complex. On the other hand, TC-NER is initiated uniquely by blockage of RNA polymerase II and subsequent recruitment of the CS-A and CS-B gene products. Thereafter, in the case of either GG-NER or TC-NER, the common “core NER pathway” is recruited to faithfully restore the integrity of the DNA through sequential steps of localized strand unwinding, incision of the DNA on either side of the adduct, excision of the adduct leaving a small single-stranded gap, and, finally, gap filling and ligation using normal DNA replication factors and the intact complementary strand as template.

The abbreviations used are: NER, nucleotide excision repair; CPD, cyclobutane pyrimidine dimer; XP, xeroderma pigmentosum; GG-NER, global genomic-NER; TC-NER, transcription-coupled NER; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; HD5, human diploid skin fibroblast; HDLF, human diploid lung fibroblast; PBS, phosphate-buffered saline; shRNA, short hairpin RNA; GST, glutathione S-transferase; XPC, XP complementation group C; XPA, XP complementation group A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PP, photoproduct.
Since its initial discovery over 40 years ago, the GG-NER pathway has been extensively studied and fully reconstituted in vitro (5). Nonetheless, relatively little is known about the potential roles in this repair process of various preeminent mutagen-responsive cellular signaling cascades. It has, however, been shown in many human cell lines treated with the model mutagen 254-nm UV (hereafter UV) that the p53 tumor suppressor, a critical stress-induced regulator of apoptosis and cell cycle checkpoints, is strictly required for efficient GG-NER (6, 7). In support of this, up-regulation of the GG-NER-specific proteins UV-DDB2 and XPC were shown to depend upon the presence of functional p53 (8, 9). Furthermore, p53 up-regulates Gadd45 and binds the histone acetyltransferase p300, events required to stimulate chromatin relaxation, which in turn facilitates access of GG-NER recognition proteins to damaged heterochromatin within the genome overall (10, 11).

Following exposure to diverse genotoxic agents, the canonical mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2), and p38 kinase, become rapidly phosphorylated and go on to activate a plethora of transcription factors that regulate apoptosis and/or growth arrest (12). Of particular interest here, it has been demonstrated in UV-irradiated human cells that all three MAPKs phosphorylate p53 on multiple amino acid residues and that this has functional consequences for both p53 stabilization and p53-dependent apoptosis (13–17). This substantial level of cross-talk between the MAPK and p53 pathways strongly suggests a priori that the former pathway may also play a role in p53-dependent GG-NER. Although previous investigations have supported such a role in human cells, this important issue has still not been conclusively addressed (see “Discussion”). We thus employed human primary and tumor cell strains wherein MAPK signaling was abrogated using highly specific small molecule inhibitors, shRNA targeting, and/or expression of dominant negative mutant protein. A sensitive flow cytometry-based NER assay recently optimized and validated in our laboratory was then used to directly determine, in each UV-irradiated human strain, whether or not individual MAPKs modulate the efficiency of DNA photoproduct removal via GG-NER.

MATERIALS AND METHODS

Cell Culture—Primary human diploid skin fibroblasts (HDSFs), including the wild type strain (GM01652B) and XPA-deficient counterpart (GM01630), were purchased from the Coriell Institute. Primary human diploid lung fibroblasts (HDLFs) were provided by Dr. J. Sedivy (Brown University). HDLF-E6 (i.e. a p53-deficient isogenic derivative stably expressing the HPV-E6 oncoprotein) was described previously (18). Low passage HDSFs and HDLFs were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, l-glutamine, and antibiotics (Wisent, Montreal, Canada). HCT116/p53+/+ human colon carcinoma cells and the isogenic p53-deficient counterpart (HCT116/p53−/−) (a gift of Dr. B. Vogelstein, The Johns Hopkins University) were cultured in McCoy’s 5A supplemented with 10% fetal bovine serum, l-glutamine, and antibiotics. Human U2OS osteosarcoma cells were grown in Dulbecco’s minimal essential medium containing 10% fetal bovine serum, l-glutamine, and antibiotics.

UV Irradiation—Cell monolayers were washed twice with PBS, covered with 2 ml of PBS, and irradiated with UV using a Philips G25T8 germicidal lamp at a fluence of 0.2 J/m2/s.

Pharmacological Inhibition of MAPK Activity in Human Primary and Tumor Cell Strains—HDSFs, HDLFs, or HCT116 colon carcinoma cells were pretreated for 2 h with M2SO (carrier) or with one among the highly specific chemical MAPK inhibitors 10 μM U0126, 30 μM SP600125, or 20 μM SB202190 (Cell Signaling Technology, Inc.) to abrogate signaling through ERK1/2, JNK1/2, and p38α/β, respectively. Following UV or mock irradiation, fresh medium containing either M2SO or inhibitor was added during subsequent incubations.

shRNA-mediated Knockdown of ERK1/2, JNK1/2, or p53 in U2OS Osteosarcoma Cells—shRNA constructs, cloned into the pLKO.1-puro vector and targeting ERK1 (clone TRCN0000006150), ERK2 (clone TRCN0000010050), JNK1 (clone TRCN0000010580), or JNK2 (clone TRCN000001012), were purchased from Sigma. ERK1 and ERK2 or JNK1 and JNK2 shRNA constructs were pooled (4 μg of each) and transiently transfected into U2OS osteosarcoma cells using Lipofectamine 2000 according to the manufacturer’s directions (Invitrogen). Immediately following antibiotic selection (i.e. for 3 days in 2 μg/ml puromycin), transfected cells were incubated for 3–5 h in complete medium without antibiotic prior to treatment with UV. Cells transfected with pLKO.1 expressing a scrambled shRNA were used as control.

For stable knockdown of p53 protein in U2OS cells, an shRNA targeting nucleotides 1095–1115 of this tumor suppressor was chosen (GenBank™ accession number AF307851). This shRNA was cloned into the pSuper-retroviral vector (OligoEngine), and infectious retroviral particles were produced following transfection of PT67 packaging cells. Retroviral transductions were carried out as previously described (19). A polyclonal U2OS population stably expressing p53-targeting shRNA was obtained following selection in 2 μg/ml puromycin (Sigma). The efficiencies of ERK1/2, JNK1/2, and p53 knockdowns were monitored by Western blotting (see below).

Abrogation of p38α/β Signaling in HDLefs via Stable Expression of the Dominant Negative Mutant p38α (AGF)—Total RNA from HDLFs was reverse transcribed using oligo(dT) primers (Invitrogen). Four μl of the reaction was used for PCR amplification of full-length human p38α wild type cDNA (GenBank™ accession number NM_001315.1) using cloned pfu polymerase (Stratagene) in conjunction with the forward and reverse primers (5’-GCTGGAAATGTCTCAGGAGA-3’ and 5’-CTCAGGACTCCATCTCTTCTT-3’), respectively. The wild type p38α cDNA was cloned into the retroviral vector pMSCVretro (Clontech). The dominant negative p38α (AGF) mutant (20) was then produced using site-directed mutagenesis according to the manufacturer’s protocol (QuikChange site-directed mutagenesis kit; Stratagene). Point mutations were introduced at each of the p38α kinase activation sites ((Thr180 (AGF) → Ala180 (ACA) → Ala180 (GCA) and Tyr182 (TAC) → Phe182 (TTT)) using the following forward/reverse primer sets (mutations underlined): 5’-CACAATGATGAAATGTCTCAGGAGA-3’ and 5’-GCCAACGTAATCGGATCATTTATC-3’.

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ATC TG TGT A-3; 5'-GAT GAA ATG GCA GCC TCT GTG GCC ACT AGG TGG-3' and 5'-CCA CCT AGT GGC CAC GAA GCC TGC CAT TTC ATC-3'). A polyclonal HDLF population stably expressing p38α (AGF) was isolated by retroviral transduction as mentioned above. Inhibition of p38α/β signaling was monitored by Western blotting (see below).

Activation and Inhibition of MAPK Signaling Determined by Western Blotting—Western blotting was performed in human primary and tumor cell strains as previously described (19). Activation or pharmacological inhibition of MAPK signaling was evaluated (i) by measuring levels of phosphorylated MAPKs using anti-phospho-ERK1/2, -JNK1/2, or -p38α/β antibodies (BIOSOURCE International) and/or (ii) by measuring levels of phosphorylated downstream MAPK effectors, where solid-phase kinase assay kits (Cell Signaling Technology, Inc.) were first used to pull down phospho-ERK1/2, total JNK1/2, or phospho-p38α/β. This was followed by phosphorilation reactions in vitro and detection of activated substrates (i.e. phospho-Erk-1, -c-Jun, or -ATF-2) using specific anti-phosphoantibodies. In order to assess p38α/β activity in vivo, as described and validated previously (21, 22), levels of phosphorylated forms of the specific p38α/β substrate MAPKAPK2 (hereafter MK2) were distinguished from and compared with unphosphorylated forms by band shift using an anti-MK2 antibody (Cell Signaling Technology Inc.). In order to verify shRNA knockdowns, levels of total ERK1/2, total JNK1/2, or p53 were probed using anti-ERK1 (Santa Cruz Biotechnology, Inc.), anti-SAPK/JNK (Cell Signaling Technology, Inc.), or anti-p53 (Santa Cruz) antibodies, respectively.

To evaluate inhibition of p38α/β signaling by expression of dominant negative p38α (AGF), MK2 phosphorylation was quantified as described above. Actin, or in some cases total JNK, was used as loading control. Bands were quantified by densitometry using MultiGauge software (Fuji Corp.).

Flow Cytometry-based Determination of NER Kinetics—Normal or XPA-deficient HDSFs were maintained at confluence for 4 days prior to irradiation (yielding >95% synchronization in G0) and maintained at confluence during post-UV incubation. In the case of HDLFS or colon carcinoma cells, exponentially growing monolayers were synchronized in G0/G1 by serum starvation (0.5%) for 3 days, and 3 h before treatment fresh complete medium was added to restimulate proliferation. U2OS cells transiently expressing shRNAs could not be synchronized by either confluence or serum starvation. As such, immediately following UV exposure, 200 ng/ml nocodazole was added to the medium to block cell division.

At various times post-UV, cells were washed with PBS plus 50 mM EDTA, trypsinized, resuspended in 1 ml of PBS plus 50 mM EDTA, and fixed by the addition of 3 ml of ice-cold 100% ethanol. 1 × 10^6 fixed cells were then washed with PBS plus 50 mM EDTA, resuspended in either 0.5% Triton X-100 plus 2 N HCl (for CPD detection) or 0.5% Triton X-100 plus 0.1 N HCl (for 6-4 photoproduct) for 20 min at 22 °C. Cells were then washed with PBS plus 300 μl of RNase (100 μg/ml in PBS) for 1 h at 37 °C followed by washing with PBS-TB (1% bovine serum albumin plus 0.25% Tween 20 in PBS). Cells were then resuspended in PBS-TB containing a primary monoclonal antibody against either CPDs or 6-4PPs (Kamiya Biomedical Company) for 1 h at room temperature, washed with PBS-TB, and then resuspended in 300 μl of fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody for 45 min at room temperature. Pellets were washed twice with PBS-TB and resuspended in 300 μl of PBS containing 5 μg/ml propidium
iodide (Molecular Probes, Inc.), and repair kinetics were monitored for populations gated in G1 using a flow cytometer fitted with an argon laser and CellQuestPro software (BD Biosciences).

Evaluation of Apoptosis, Clonogenic Survival, and Cellular Proliferation in HDLFs—At various times post-UV, in HDLFs treated or not treated with MAPK inhibitors, apoptosis was investigated by both (i) annexin-V labeling using a kit according to the manufacturer’s directions (Sigma) and (ii) sub-G1 peak analysis following staining of cells with propidium iodide. Annexin-V-positive early apoptotic cells or propidium iodide-positive sub-G1 cells were quantified using a FACScan flow cytometer fitted with CellQuestPro software (BD Biosciences). For determination of clonogenic survival, appropriate cell numbers were seeded on 100-mm dishes 6 h prior to treatments with or without MAPK inhibitors. Cells were incubated for 15–21 days, and colonies were scored after staining with 0.5% methylene blue in 50% methanol. Cellular proliferation was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a kit as described by the manufacturer (American Type Culture Collection).

RESULTS

UV-induced Activation and Pharmacological Abrogation of MAPK Activity in Human Cells—Replicate cultures representing three human strains (i.e. to stringently control for cell type-specific effects that are highly characteristic of MAPK function) were irradiated with 10 J/m² UV (yielding ~5–10% relative clonogenic survival in each strain; data not shown) in the presence of either carrier (Me₂SO) or well characterized, highly specific chemical MAPK inhibitors (i.e. U0126, SP600125, and SB202190) to abrogate signaling through ERK1/2, JNK1/2, and p38α/β, respectively) (21, 23, 24). At various times post-UV, cultures were evaluated for levels of MAPK phosphorylation. In the case of HDSFs, phospho-JNK1/2 and phospho-p38α/β peaked at early times (0.5–1 h) and diminished rapidly thereafter, whereas phospho-ERK1/2 peaked at 0.5 h and remained elevated for at least 24 h (Fig. 1A). This correlated temporally with in vitro phosphorylation by phospho-ERK1/2, JNK1/2, and -p38α/β of the specific downstream effectors Elk-1, c-Jun, and ATF2, respectively (Fig. 1B). Furthermore, incubation of HDSFs with chemical inhibitors resulted in strong diminishment (>85%) of ERK1/2, JNK1/2, or p38α/β activity at 1 h post-UV (Fig. 1, C–E). Results similar to those described immediately above for MAPK activation/abrogation in HDSFs were obtained for UV-irradiated HDLFs (Fig. 2, A and B). However, in the case of HCT116/p53<sup>−/−</sup>, although phospho-JNK1/2 peaked at 1 h post-UV as anticipated, ERK1/2 appeared to be constitutively phosphorylated (Fig. 2, C and D), and no phospho-p38α/β could be detected within 48 h despite the presence of nonphosphorylated forms (not shown).

Optimization and Validation of a Flow Cytometry-based DNA Repair Assay and Its Application to Examine the Potential Role of MAPK Signaling in Human GG-NER—In parallel with the above MAPK activation/abrogation studies, cultures were set aside at various times post-UV to evaluate the role of individual MAPKs in GG-NER using a flow cytometry-based DNA repair assay recently developed in our laboratory. This assay directly detects CPDs in partially denatured double-stranded DNA in intact permeabilized cells using highly specific fluorescently labeled anti-CPD antibodies. Although a similar method was previously reported over a decade ago (25), it was subsequently only very rarely used to address biological mechanisms.
We have now optimized this type of assay (i) to be rapid, reproducible, and extremely sensitive, detecting CPDs induced by UV doses as low as 1 J/m² and (ii) to exhibit a linear dose response for CPD induction up to 40 J/m² (Fig. 3A). Our optimized NER assay was initially applied to confluent UV-exposed HDFSs treated or not with MAPK inhibitors. (For all repair analyses described in this study, cells were synchronized by various approaches (see “Materials and Methods”) to prevent cell division, which would otherwise dilute the CPD-specific immunofluorescence signal, thereby interfering with accurate determination of repair kinetics.) Representative raw data are shown for normal HDFSs and NER-deficient XPA HDFSs (Fig. 3, B and C), and the complete experiment in HDFSs is graphically illustrated in Fig. 4A. Relative to Me₂SO-treated controls, incubation with any of U0126, SP600125, or SB202190 had virtually no effect on CPD removal over a 48-h period in normal HDFSs, whereas GG-NER-deficient XPA HDFSs (negative control), in line with expectations, exhibited almost complete abrogation of CPD repair. For HDLFs, precisely the same trends with respect to CPD repair kinetics were observed (Fig. 4B). Also, repair of another UV-induced bulky adduct, i.e. the 6-4PP (induced 5-fold less frequently than CPDs), was evaluated in HDLFs after irradiation with 25 J/m² UV using a specific anti-6-4PP antibody. 6-4PPs are observed (Fig. 4A). Also, repair of another UV-induced bulky adduct, i.e. the 6-4PP (induced 5-fold less frequently than CPDs), was evaluated in HDLFs after irradiation with 25 J/m² UV using a specific anti-6-4PP antibody. 6-4PPs are additionally, as fully expected, HPV-E6-expressing HDLFs and XPA-deficient HDLFs (negative control) treated or not with MAPK inhibitors, each time point represents the mean ± S.E. of at least three independent experiments. DMSO, Me₂SO.

FIGURE 3. Validation of a novel flow cytometry-based NER assay. A, CPD induction in normal HDFSs as a function of dose. B and C, representative raw profiles depicting CPD repair kinetics for normal HDFSs and for NER-deficient XPA HDFSs, following UV exposure. The arrows indicate small artifactual shoulders in the flow cytometry curves, ostensibly generated by minor populations of doublet cells, which were routinely excluded from repair analysis.

FIGURE 4. Flow cytometry-based evaluation of NER kinetics in UV-exposed human primary and tumor cell lines treated with chemical MAPK inhibitors. A, kinetics of CPD removal in UV-exposed normal HDFSs and in UV-exposed XPA-deficient HDFSs (negative control), treated or not with MAPK inhibitors. B, kinetics of CPD removal in UV-exposed HDLFs and in isogenic UV-exposed HDLF-E6 cells (negative control), treated or not with MAPK inhibitors. C, kinetics of 6-4PP removal in UV-exposed HDLFs and in UV-exposed XPA-deficient HDFSs (negative control), treated or not with MAPK inhibitors. D, kinetics of CPD removal in UV-exposed human HCT116 p53⁻/⁻ colon carcinoma cells and in UV-exposed p53-deficient isogenic counterparts (HCT116 p53⁻/⁻; negative control), treated or not with MAPK inhibitors. Each time point represents the mean ± S.E. of at least three independent experiments. DMSO, Me₂SO.
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characteristically exhibited significantly reduced GG-NER efficiency relative to its isogenic p53-proficient counterpart.

To reinforce the above data obtained in human primary or tumor cells treated with highly specific chemical MAPK inhibitors, human U2OS osteosarcoma cells were transiently transfected with shRNAs targeting either total ERK1/2 or total JNK1/2, followed by evaluation of GG-NER kinetics. As negative control for these studies, U2OS cells stably transduced with a retroviral vector expressing shRNA against p33 were employed. U2OS cells, like other cell lines evaluated in this study, exhibited maximal phosphorylation of JNK1/2 and of ERK1/2 by 0.5–1 h following irradiation with 10 J/m² UV (Fig. 5A). Transient transfection of U2OS with shRNAs targeting total ERK1/2 or total JNK1/2 or stable expression of an shRNA targeting p53 reduced protein expression by at least 70% (Fig. 5B). Nonetheless, consistent with our repair data using chemical MAPK inhibitors, no significant effect of ERK1/2 or JNK1/2 knockdown on GG-NER efficiency was observed (Fig. 5C). As fully expected, however, shRNA-mediated knockdown of p53 in U2OS cells significantly inhibited CPD removal.

In addition to the shRNA experiments in U2OS cells described above, HDLFs were stably transduced with a retroviral vector expressing the dominant negative p38α (AGF) mutant. Expression of this mutant, compared with HDLFs expressing an empty vector, resulted in a 5-fold reduction in the ratio of phosphorylated to unphosphorylated forms of the unique p38α/β substrate MK2 (Fig. 5D, top). Despite this indication that p38α/β signaling had been substantially reduced, no significant effect on the kinetics of GG-NER was observed (Fig. 5D, bottom), although HDL-F-E6 cells, as expected, manifested a significant reduction in GG-NER capacity.

Effects of MAPK Inactivation on Cell Death and Proliferation in Primary Human Lung Fibroblasts—Having observed no effects on GG-NER as above, it became necessary to provide assurance that in our hands abrogation of MAPK signaling nonetheless generates some anticipated phenotypic outcomes. We therefore undertook to characterize the effects of MAPK inhibitors on cell death and proliferation in HDLFS, either unstimulated or exposed to UV. No reports to our knowledge have previously addressed such MAPK-dependent phenomena in primary human cells from the lung. In this sense, it should be emphasized that in general DNA damage responses elicited by MAPKs are subject to high variability (e.g. although JNK1/2 activation is most often associated with induction of apoptosis, it is now becoming increasingly clear that this kinase can mediate either proapoptotic or antiapoptotic effects, or no apoptotic effects, depending upon stimulus, dose, and/or cell type) (27, 28). Specifically in the case of HDLFS irradiated with 10 J/m² UV, SP600125 treatment significantly stimulated apoptosis at 48 and 72 h post-UV as evaluated by either sub-G1 peak analysis or annexin V staining (p < 0.05; two-tailed unpaired t test) (Fig. 6, A and B). These data support an antiapoptotic role for JNK1/2 in UV-exposed HDLFs, in line with prior investigations in some
other cultured cell strains (29, 30). On the other hand, perhaps not surprisingly based on a dearth of previous studies to the contrary, no effect on UV-induced apoptosis was noted here upon ERK1/2 inactivation in HDLFs (Fig. 6, A and B). Finally, we observed a moderate but significant decrease in annexin V-positive staining at 72 h post-UV in SB202190-treated HDLFs (Fig. 6B), supporting a proapoptotic role for p38α/β in UV-irradiated HDLFs, as previously observed in various cell lines (31). In addition, sub-G1 peak analysis consistently revealed a slight reduction in UV-induced apoptosis in SB202190-treated cells, although this was not statistically significant (Fig. 6A).

Clonogenic survival and cellular proliferation by MTT assay were also evaluated in HDLFs treated or not with MAPK inhibitors. In line with previously reported tendencies in tumor cell lines (28, 32–35), abrogation of MAPK signaling in either mock- or UV-irradiated primary lung fibroblasts (i) significantly reduced long term colony-forming ability (Fig. 6C) and (ii) negatively impacted cellular proliferation by 96 h post-UV (Fig. 6D).

DISCUSSION

The current study provides solid evidence that abrogation of ERK1/2, JNK1/2, or p38α/β activity in UV-exposed human primary and tumor cells via treatment with highly specific chemical inhibitors, although significantly influencing cell death and proliferation, has no effect whatsoever on the efficiency of GG-NER. This conclusion is strongly reinforced by experiments showing that repair is similarly unaffected following (i) transient shRNA-mediated knockdown of ERK1/2 or JNK1/2 in U2OS osteosarcoma cells or (ii) stable expression of a dominant negative p38α mutant in HDLFs. We strongly emphasize that the observed lack of participation of MAPK signaling in human GG-NER is unexpected in light of previous reports. For example, UV-irradiated transformed mouse embryonic fibroblasts, either genetically null for the JNK1/2 effector c-fos or treated with SP600125, were shown to be deficient in GG-NER as determined by Southwestern blotting analysis using a specific anti-CPD antibody (29); furthermore, interference with c-Fos-mediated up-regulation of XPF (i.e. a structure-specific endonuclease required to incise DNA at damaged sites during NER) may underlie the observed effect (36). The apparent discrepancy between these data in a murine system and ours in humans may be attributable to species-specific effects. In fact, rodent cells, relative to human, generally exhibit profoundly reduced expression of the GG-NER-specific protein UV-DDB2 and are consequently much less proficient in GG-NER (37), a significant caveat that may complicate interspecies comparisons.

Regarding previous reports in human cells, interference with JNK1/2 signaling was shown to engender cellular sensitivity to the UV-mimetic agent cisplatin as well as decreased post-treatment recovery of PCR amplification efficiency (DNA polymerase stop assay) (35, 38, 39). Although it was legitimately concluded that this might be attributable to deficient removal of cisplatin-induced DNA adducts, the affected repair pathway(s) could not be identified via the indirect polymerase stop assay employed. Indeed although cisplatin induces replication-blocking DNA intrasstrand cross-links that are repaired by NER, this agent also generates a highly significant yield of replication-blocking DNA interstrand cross-links. The latter type of cross-link
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represents a distinct class of adduct that requires (in addition to NER) the concerted action of multiple DNA repair pathways for efficient removal (i.e., homologous recombination, DNA mismatch repair, and DNA translesion synthesis) (40), any of which may be influenced by MAPK signaling. On the other hand, herein we have monitored DNA adduct repair in intact cells by direct quantification of CPDs or 6-4PPs, both of which are clearly removed exclusively by NER.

It is important to highlight another recent report that contributes to the burgeoning perception that MAPKs participate in the NER process. Specifically, ERK1/2 signaling was firmly linked to regulation of the essential NER core pathway protein ERCC1 in UV-exposed human hepatoma cells, and additionally, abrogation of ERK1/2 activation with U0126 was shown to possibly reduce the efficiency of UV DNA photoproduct removal as evaluated by a host cell reactivation assay (41). However, again (i.e. analogous to the situation for the aforementioned DNA polymerase stop assay) such an experimental approach, which measures post-UV recovery of plasmid-borne reporter gene expression, only indirectly reflects repair of DNA polymerase II-blocking lesions. Other caveats associated with the above study include the following: (i) DNA repair kinetics were not evaluated (i.e. recovery of transcription was examined only at 24 h post-UV), and curiously (ii) the putative effect of ERK1/2 on NER was observed following irradiation with 80 J/m² UV but not with 40, 60, or 120 J/m².

It should be noted that although MAPKs apparently do not influence repair of CPDs within the genome overall via GG-NER in human cells, such an influence on repair along the transcribed strands of active genes via TC-NER (which is not measured by our GG-NER assay) cannot be entirely ruled out. Indeed, it is possible that MAPKs participate in TC-NER but not GG-NER by regulating the unique lesion recognition step of the former NER subpathway, involving, for example, activation of the TC-NER-specific proteins CS-A or CS-B and/or removal of stalled RNA polymerase II at damaged sites and subsequent recruitment of the core NER pathway.

The MAPKs comprise a preeminent mutagen-inducible cascade, which, by all previous indications, appeared to play a significant role in GG-NER. Nonetheless, here we have employed a highly sensitive DNA repair assay to directly demonstrate that MAPKs play no such role in cultured human cells. These data thus reorient our mechanistic perception of a critical antineoplastic DNA repair pathway. We also emphasize that aberrant MAPK signaling has been firmly implicated in diverse pathologies, including cancer, inflammation, and cardiovascular disorders; furthermore, MAPK inhibitors are currently being evaluated in therapy for such diseases (42, 43). The revelations provided by our study would ostensibly constitute an important consideration when designing treatment protocols that target the MAPK pathway.

Acknowledgments—We gratefully acknowledge Drs. Dindial Ramotar and Eric Milot for critically reading the manuscript.

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