p38 MAPK Activation Selectively Induces Cell Death in K-ras-mutated Human Colon Cancer Cells through Regulation of Vitamin D Receptor*

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The Ras family of proteins consists of three isoforms, H-, K-, and N-Ras, which play critical roles in control of normal and transformed cell growth (1, 2). Mutated K-ras is one of the most frequent genetic events in human cancer, with the highest incidences in pancreatic carcinomas (90%) and colorectal tumors (50%) (3, 4). Various strategies have been attempted to selectively inhibit oncogenic Ras activity, including application of farnesyltransferase inhibitors (5, 6), antisense technology (7–9), and small interference RNA (10). Although certain progress has been made in experimental models, therapeutic strategies to selectively inhibit activated ras oncogenes in human cancer remain to be established.

Increasing knowledge of Ras signaling in recent years has suggested an important alternative for the inhibition of oncogene Ras activity through regulation of Ras downstream signal transduction pathways (11). Multiple pathways are involved in transduction of Ras signaling, among which the best characterized are the mitogen-activated protein kinases (MAPKs),1 including extracellular mitogen-regulated kinase (ERK), c-Jun amino-terminal kinases (JNK), and p38 (12). Of these MAPKs, the Ras/Raf/MEK/ERK pathway is most critical in Ras-induced proliferation and transformation (13). Activation of this cascade has been shown to be both necessary and sufficient for Ras transformation (14–17). Accordingly, efforts have been made to inhibit this key pathway with specific MEK inhibitors to explore the therapeutic potential (18, 19). The MEK/ERK pathway, however, is also required for normal cellular proliferation in response to growth factors. Consequently, the usefulness of the inhibition of MEK/ERK activity to suppress the ras oncogene-induced cancers remains to be further verified.

In addition to the mitogenic ERK pathway, Ras also signals to the JNK and p38 stress MAP kinases (13). Oncogene ras is known to activate JNK and its physiological substrate c-Jun (20, 21). The JNK pathway was also shown to be required for Ras transformation in many cell types because JNK inhibition suppresses Ras-induced transformation (22), and Ras-transforming activity is compromised in c-Jun knock-out cells (23). In contrast to the cooperative role of the JNK pathway, our previous work demonstrated that the p38 MAPK pathway is inhibitory in Ras mitogenic signaling (24). This was shown by the fact that oncogenic Ras activates p38 and its downstream kinases PRAK and MK2 in NIH 3T3 cells, and each of these kinases in turn suppresses Ras-induced proliferation (24). The inhibitory effect of p38 kinase on Ras activity was further confirmed in rat intestine epithelial cells in which the p38 inhibition by SB drugs leads to an increased growth of Ras-transformed cells in soft agar (25). Moreover, the p38 activation in human mammary epithelial cells appeared to be specific to H-Ras but not to N-Ras (26), and higher levels of phosphorylated p38 proteins were even shown to be able to predict an in

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; VDR, vitamin D receptor; Luc, luciferase; ARS, arsenite; pSR, pSUPER, Vect, vector.
**p38 Selectively Kills K-ras-mutated Human Colon Cancer Cells**

**MATERIALS AND METHODS**

**cDNA Constructs and Cell Lines**—The construct of the recombinant adenovirus vector containing HA-tagged constitutively active MKK6 (ad-MKK6) was prepared as previously described (29, 30). Human VDR cDNA was provided by Leonard Freedman (31) and was cloned into a V5-tagged pDNA3 expression vector (Invitrogen). A FLAG-tagged constitutively active p38 activator MKK6 in a pDNA3 vector was previously described (32). A 0.5-kb mouse VDR luciferase promoter (VDR-Luc) was provided by Hector DeLuca (33). The AP-1 site-mutated VDR promoter was characterized previously in our laboratory (29). The AP-1 luciferase reporter (AP-1 Luc, three AP-1 repeats fused to a minimal c-Fos promoter) was provided by Craig Hauser (34). Human colon cancer cell line HCT116 containing a mutated K-ras at codon 13 (Gly to Asp) and its K-ras-disrupted subclones, HKe3 and HK2–, have been described (35–37). Human colon cancer cells SK-CO-1 (K-ras-mutated) and HT-29 (without K-ras mutation) were provided by Dennis Stacey (38, 39).

**Cell Culture, Transfection, and Luciferase Assay**—Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum at 37 °C, and 5% CO2. To establish stable VDR expression clones, HCT116 cells were transfected with a V5-tagged VDR construct or a corresponding vector using calcium phosphate (a kit from Promega) and selected with G418 for drug-resistant clones (40). For AP-1 and VDR luciferase promoter assay, cells were transiently transfected with AP-1 Luc or VDR-Luc, with or without cotransfection with MKK6, and luciferase activity was determined 48 h after transfection using a dual luciferase kit from Promega as previously described (29). In the case of ARS treatment, the reporter assay was carried out 24 h after the pulse treatment.

**Experiments with Small Interference RNA to Inhibit Endogenous VDR Expression**—To silence endogenous VDR protein expression, a pSuper (pSR) RNAi kit (catalogue number VEC-pRT-0002; OligoGene) was used. This retrovirus pSR vector is designed to direct transcription from a pair of 64-nt oligonucleotides, which are processed into functional small interference RNA to specifically inactivate sequence, downstream of the start codon of the human VDR gene (coding region) and designed four corresponding 64-mer oligonucleotides (synthesized by IDT Company). These oligonucleotides were cloned into the pSR vector according to the manufacturer's protocol. For infection, the pSR constructs were transfected into the Phoenix-Ampo retrovirus packaging cells (ATCC) as previously described (41). Two days after the transfection, supernatants were used to infect HKe3 colon cancer cells. One of these sequences (5′-GATGATCCAGAAGCTAGCC-3′ at 1259) in pSR was shown to consistently suppress endogenous VDR protein expression in 293 as well as HKe3 colon cancer cells and was used for the toxicity assay.

**Cell Death Assay**—For cell death, cells were plated at the same density and treated with 20 μM ARS or 40 μM amascerine (provided by NCI, National Institutes of Health) for 24 h. Infection with adenovirus vector and adenovirus containing MKK6 was carried out as previously reported (29), and cell death was analyzed 48 h following infection. Cell death was determined by the trypan blue exclusion assay and analyzed by Student’s t test for statistical significance. Results from the viability assay were confirmed either by morphological observations under light microscope and/or fluorescence-activated cell sorter for a sub-G0 population (30, 42).

**Western Blotting Analyses**—Cells were pulsed-treated with ARS for 30 min and collected in 1× loading buffer to examine for protein expression and protein-phosphorylation. In the case of adenovirus infection, cells were collected 24 h postinfection for Western analyses. Protein was separated on an SDS-PAGE, which was transferred to a nitrocellulose membrane using a liquid-transfer system (Bio-Rad). The resultant membrane was typically blocked with 5% milk in Tris-buff-ered saline-Tween 20 for 1 h and incubated with primary antibody in 5% milk TBST at 4 °C overnight. Rabbit and goat polyclonal antibodies against VDR (C-20), c-Jun, and ATF-2 were purchased from Santa Cruz Biotechnology. Antibodies against phosphorylated p38, JNK, ERK, c-Jun (Ser-63), and ATF-2 and their corresponding forms for total proteins were from Cell Signaling. The membrane was then washed four times with 1× TBST and incubated with the proper peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The ECL reaction was carried out according to the manufacturer’s instructions (Amersham Pharmacia Biotech), and the resultant bands were visualized in a PhosphorImager (Amersham Bioscience). Each membrane was typically stripped off and reprobed with additional antibodies for comparison and normalization as described previously (29). Similar results were obtained from at least one additional experiment.

**RESULTS**

**p38 Activation Selectively Induces Cell Death in Human Colon Cancer Cells with K-ras Activation**—To examine effects of p38 activation on cell survival of human colon cancer cells with and without endogenous Ras activation, the K-ras-activated HCT116 human colon cancer cell line and its two K-ras-disrupted subclones (HKe3 and HK2–) were treated with p38 activators. Previous work has demonstrated that disruption of the activated K-ras gene in HCT116 cells by homologous recombination leads to a loss of malignant growth in soft agar and nude mice (35). To activate p38 kinase, cells were infected with an adenovirus vector containing a constitutively active MKK6 (ad-MKK6), a specific p38 activator (28, 32, 43), or a control empty vector (Vect). Moreover, a group of cells was also treated with a typical physiological chemical p38 stimulus, ARS (24, 44), to confirm results with MKK6 without cDNA overexpression. As shown in Fig. 4A, both ARS and MKK6 selectively induced cell death in HCT116 cells that harbor an activated K-ras (p < 0.05 versus control), but the same treatment had no substantial effects on cell viability in K-ras-disrupted HK2– and HKe3 subclones (p > 0.05 versus Vect control in both lines). Although infection with ad-MKK6 led to a moderate increase in sub-G1 population as well as some morphological alterations in HKe3 and HK2– cells (Fig. 1, B and C), these effects are much less than those in K-ras-activated HCT116 cells. Hence, MKK6 and ARS are specifically toxic to K-ras-activated human colon cancer cells.

To analyze effects of MKK6 and ARS on p38 activity, cell lysates were prepared and analyzed by Western blot for p38 phosphorylation using a specific p38 phospho-antibody and compared with levels of phosphorylated ERK and phosphorylated JNK proteins. Also, the effects on c-Jun and ATF-2, transcription factors downstream of the JNK and p38 kinases that are most frequently activated by stress signaling (45–47), were examined. Infection with ad-MKK6 selectively induced p38 phosphorylation without substantial effects on the JNK and ERK activity. These effects occurred similarly in all three cell lines regardless of the Ras status (Fig. 2). Moreover, MKK6 had no effect on c-Jun activity but activated ATF2 in all three cell lines. ATF2 activation was shown as an increased band density as well as a decreased mobility as a result of phosphorylations (48). The similar effects of MKK6 on p38 and p38 substrates ATF-2 and c-Jun (49) in all three cell lines suggest that the cell death-inducing effect of MKK6 is not due to its selective activation of the p38 pathway in HCT116 cells.

Although ARS treatment similarly stimulates p38 phosphorylation in three cell lines, this chemical, as opposed to MKK6, also activates JNK and ERK, which are more predominant in HCT116 cells in comparison with the K-ras-disrupted lines (Fig. 2). Moreover, ARS appears to specifically phosphorylate c-Jun in K-ras-activated HCT116 cells, an effect that is opposite to that induced by mitogenic stimuli such as serum and 12-O-tetradecanoylphorbol-13-acetate, which more strongly ac-
apoptotic genes through a combined effect of K-ras mutation and p38 activation.

p38 Activation Induces an AP-1-dependent Trans-suppression of VDR Gene Expression in K-ras-activated but Not in K-ras-disrupted Colon Cancer Cells—VDR is a member of the nuclear receptor superfamily of ligand-activated transcription factors, which is believed to mediate most, if not all, biological effects of vitamin D3 (53). Although VDR is traditionally considered only to be expressed in vitamin D3 target tissues such as skin, intestine, and kidney (54), the VDR transcript and/or its protein product is actually detectable in most of the tissues/cell types examined (53). Our recent work established that VDR in breast cancer cells is trans-activated by a combined AP-1 signal induced by the p38 and JNK stress MAP kinases in a manner independent of ligand vitamin D3 (29). This observation indicates that VDR may perform previously unappreciated functions in stress response. To explore whether MKK6- and ARS-induced cell death in HCT116 cells is through the regulation of VDR, p38 activation-regulated VDR protein expressions were determined by Western blot analysis. Of interest, both ad-MKK6 infection and ARS treatment led to a significant reduction of VDR protein expression in the activated Ras-containing HCT116 cells (Fig. 3A). The reduction of VDR protein levels is in contrast to MKK6 and ARS stimulation of VDR in human breast cancer cells (29). In the colon cancer HCT116 cells, infection with ad-MKK6 resulted in a ~50% decrease in VDR protein expression (49.4% ± 6.9% of the vector control from three separate experiments). More interestingly, the same treatments had no observable effects on VDR protein levels in K-ras knock-out Hke3 cells (Fig. 3B). The different effects of ad-MKK6 on VDR in HCT116 and Hke3 cell lines are not due to different MKK6 protein expression after adenovirus infection (Fig. 3C). These results thus demonstrated that MKK6 and ARS are specifically inhibitory to VDR protein expression in K-ras-mutated human colon cancer cells.

Because MKK6 activates VDR through an AP-1 site in the VDR promoter in human breast cancer cells (29), we were then interested to explore whether the difference in VDR regulation is because of different signals from the AP-1 transcription activity. To this end, AP-1-dependent transcription was determined by transient transfection of an AP-1 luciferase reporter (AP-1 Luc) with and without MKK6, and the luciferase activity was determined 48 h after transfection. Results in Fig. 4A showed that MKK6 reduces AP-1 activity in HCT116 cells (p
<0.05), but not in Hke3 cells. The AP-1 inhibitory effect in HCT116 cells is specific to p38 activation by MKK6 because a JNK activator, MEKK1, stimulated AP-1 in both cell lines (data not shown). Together, these results indicate that the AP-1 inhibitory effect of MKK6 in K-ras-activated cells represents differences in p38 pathway signal integration at AP-1 that is specifically associated with K-ras activation. It is thus critical to explore further whether this different AP-1 transcriptional activity in cells with and without K-ras activation can lead to a changed regulation of the AP-1 target gene VDR.

To examine whether the VDR down-regulation occurs through AP-1-dependent transcription, a 0.5-kb mouse VDR luciferase promoter (Wt-AP1-VDR) (33) and its AP-1 site-mutated counterpart (Mt-AP1-VDR) were transfected into HCT116 cells. Effects of MKK6 and ARS on the VDR promoter activity were examined by luciferase assay. As shown in Fig. 4B, both MKK6 and ARS decreased the wild-type VDR promoter activity in K-ras-activated HCT116 cells. More strikingly, this suppressing effect was completely abolished when the AP-1 site on the promoter was mutated (29). Consistent with VDR protein regulations, MKK6 and ARS have no significant effects on the VDR promoter activity in K-ras-disrupted Hke3 cells (Fig. 4C). These results together suggest that it is a combined effect of K-ras mutation and p38 activation that leads to an AP-1 inhibition, resulting in VDR trans-suppression.

Levels of VDR Protein Concentration in Human Colon Cancer Cells Determine Their Sensitivity to p38-induced Cell Death—To directly examine whether VDR down-regulation is responsible for the selective toxicity of p38 activation in HCT116 cells, human VDR cDNA in a V5-tagged pcDNA3 vector was forced to express in these cells by stable transfection and G418 selection. Drug-resistant clones were pooled and examined for V5-VDR expression by Western blotting in comparison with the empty vector-transfected cells (Fig. 5A, 116/Neo). Early passages of these cells were transfected with MKK6 or ARS, and cell death was examined as described in Fig. 1. Results in Fig. 5B showed that VDR overexpression significantly reduced cell death by MKK6 or ARS, and similar cell death protection was also observed against another p38 stimulant, amsacrine (data not shown). Although vitamin D3 is known to be inhibitory in colon cancer cell proliferation, perhaps through a differentiation-associated process (55–57), addition of vitamin D3 to these cells did not significantly alter their sensitivity to MKK6 or ARS (data not shown). These results suggest a ligand (vitamin D3)-independent anti-apoptotic function of VDR in human colon cancer cells. Although additional signaling components may also be involved in p38-induced cell death, these results suggest that VDR down-regulation represents one important mechanism by which K-ras-activated human colon cancer cells are selectively targeted.

If cellular VDR concentrations determine the outcome of p38 activation in colon cancer cells, depletion of endogenous VDR protein in K-ras-disrupted cells should increase their sensitivity to p38 pathway-induced cell death. In this case, a retrovirus vector (pSR)-mediated delivery of the VDR small interfering RNA was used to knock down endogenous VDR protein (VDRi) in K-ras-disrupted Hke3 cells (40). Infection with pSR-VDRi led to about 80% depletion of VDR protein in comparison with the control pSR vector infection (Fig. 5C). Strikingly, the VDR depletion resulted in a significant increase in the sensitivity of K-ras-disrupted Hke3 cells to ARS-induced cell death (Fig. 5D, p <0.05 versus the pSR vector-infected cells treated with ARS). ARS was used as the p38 pathway activator in these experiments, because ad-MKK6 infection would necessitate an addi-
trypan blue staining (Fig. 6). Following ad-MKK6 infection, cell viability was determined by trypan blue staining. Results shown are the mean of four experiments (bars, S.E.; p < 0.01).

This may occur through a combined effect of Ras and MKK6 (ARS) to suppress VDR protein expression/activation.

**DISCUSSION**

Our results have presented several pieces of evidence that together support a model that p38 stress pathway activation selectively induces cell death in K-ras-activated human colon cancer cells through VDR regulation (Fig. 7). First, p38 activation, either by genetic or chemical means, selectively induces cell death in K-ras-mutated HCT-116 and SK-CO-1 cells, but not in K-ras-disrupted HCT116-derived Hke3 and HK2-8 cells or in colon cancer HT-29 cells that contain normal K-ras alleles. Second, mechanistic analysis revealed that the selective cell death-inducing effect is linked to a lower level of VDR protein concentrations. In HCT116-derived cell lines, the selective toxicity of p38 activation by MKK6 and ARS correlates with an AP-1-dependent VDR trans-suppression, whereas in K-ras-activated SK-CO-1 cells the increased sensitivity associates with the lack of VDR induction by MKK6 in comparison with HT-29 cells that contain normal K-ras genes. A cell death inhibitory activity of VDR is further confirmed by a protection against p38-induced cell death by forced VDR expression in K-ras-activated HCT-116 cells and by an increase of ARS-induced toxicity in K-ras-disrupted Hke3 cells through the small interfering RNA-mediated depletion of endogenous VDR protein. Hence, in addition to proliferative signaling (35), activated K-ras also triggers VDR down-regulation or prevents its activation in response to p38 activation, which may serve as a suicide pathway in human colon cancer cells.

Demonstration of the selective toxicity of p38 activation in K-ras-activated HCT116 human colon cancer cells through VDR inhibition contributes to our understanding of the increased sensitivity to stress-induced cell death in Ras oncogene signaling. An increased apoptotic response to ceramide was previously demonstrated in the HCT116 line in comparison with the K-ras knock-out Hke-3 cells, but the mechanism was not established (36). Although an increased sensitivity of NIH 3T3 cells transformed by ras and other oncogenes to stress has been reported by us (42) and others (59, 60), most of these...
The selective cell killing effects of p38 activation in K-ras-mutated human colon cancer cells may have important therapeutic implications. Reagents that activate the stress p38 pathway may specifically induce cell death in human colon cancer cells with a mutated K-ras oncogene without affecting survival of colon cancer cells that contain normal cellular K-ras genes. Theoretically, any agents that activate p38 kinase could have therapeutic value in treatment of K-ras-activated human colon cancer. These reagents, however, also activate additional signaling cascades besides p38 MAPK in a manner that is dependent on cell type. For example, although MKK6 was shown to specifically activate p38 in HCT116 colon cancer cells, it also stimulates JNK in human breast cancer cells (29). However, many of the corollary effects of these compounds, such as activation of ERK and JNK, may be compensated by each other in the determination of the cellular outcome (72, 73) and consequently may have no biological consequence. Therefore, experiments are warranted to further examine the selective toxicity of additional p38 stimuli, including genetic and chemotherapeutic agents (74, 75), in a panel of K-ras-activated human cancer cell lines.

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