Protein Kinase C Delta Induces Apoptosis of Vascular Smooth Muscle Cells through Induction of the Tumor Suppressor p53 by Both p38-dependent and p38-independent Mechanisms*

Received for publication, July 1, 2005, and in revised form, August 11, 2005 Published, JBC Papers in Press, August 23, 2005, DOI 10.1074/jbc.M507187200

Evan J. Ryer1, Kenji Sakakibara1, Chunjie Wang1, Devanand Sarkar1, Paul B. Fisher1,5, Peter L. Faries4, K. Craig Kent1, and Bo Liu1,2

From the 1Department of Surgery, Division of Vascular Surgery, New York Presbyterian Hospital and Weill Medical College, Cornell University, New York, New York 10021 and the 2Departments of Pathology, Neurosurgery, and Urology, Herbert Irving Comprehensive Cancer Center, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Apoptotic death of vascular smooth muscle cells (SMCs) is a prominent feature of blood vessel remodeling. In the present study, we examined the novel PKC isoform protein kinase C delta (PKCδ) and its role in vascular SMC apoptosis. In A10 SMCs, overexpression of PKCδ was sufficient to induce apoptosis, whereas inhibition of PKCδ diminished H2O2-induced apoptosis. Moreover, evidence is provided that the tumor suppressor p53 is an essential mediator of PKCδ-induced apoptosis in SMCs. Activation of PKCδ led to accumulation of p53 as well as phosphorylation of p53 in SMCs; this induction correlated with apoptosis. Furthermore, blocking p53 induction with small interference RNA or targeted gene deletion prevented PKCδ-induced apoptosis, whereas restoring p53 expression rescued the ability of PKCδ to induce apoptosis in p53 null SMCs. We also establish that PKCδ regulates p53 at both transcriptional and post-translational levels. Specifically, the transcriptional regulation required p38 MAPK, whereas the post-translational modification, at least for serine 46, did not involve MAPK. Additionally, PKCδ, p38 MAPK, and p53 co-associate in cells under conditions favoring apoptosis. Together, our data suggest that SMC apoptosis proceeds through a pathway that involves PKCδ, the intermediary p38 MAPK, and the downstream target tumor suppressor p53.

Apoptosis of vascular smooth muscle cells (SMCs)3 is a well-established component of the remodeling that occurs during normal development of the circulatory system (1, 2) as well as during the course of neointimal formation after intervention for atherosclerosis (3–5). Because increased total cellularity is a prominent feature of an occluding neointima, the balance between proliferation and apoptosis during vessel healing appears central to this pathologic process (6, 7). Indeed, accumulating evidence suggests that abnormal SMC apoptosis leads to neointimal hyperplasia (8–11). However, despite the importance of SMC apoptosis, the precise molecular mechanism underlying the regulation of apoptotic pathways in SMCs remains largely undetermined.

Apoptosis is a multistage, genetically controlled process of selective cell deletion. Protein kinases regulate the early stages of apoptosis by phosphorylating key proteins (12, 13), whereas caspases, a family of cysteine proteases, are the main effectors whose activation results in the characteristic morphological changes associated with programmed cell death such as membrane blebbing, chromatin condensation, and DNA fragmentation (14, 15).

Members of the protein kinase C (PKC) family are activated by diverse stimuli and participate in multiple cellular processes such as growth, differentiation, and apoptosis (16). The novel PKC isoform, protein kinase C delta (PKCδ), has been shown to be associated with the response to DNA damage and other apoptotic stimuli in specific cell types (17–20). The critical role of PKCδ in vascular SMC apoptosis and pathogenesis of a neointimal lesion has been recently demonstrated using PKCδ “knock-out” mice. The PKCδ null mice developed exacerbated vein graft intimal lesions that contain fewer apoptotic vascular cells compared with the wild-type mice (21). Furthermore, aortic SMCs isolated from PKCδ null mice are resistant to apoptotic stimuli including H2O2. However, the mechanism by which PKCδ mediates SMC apoptosis remains to be defined.

The tumor suppressor p53 is the master regulator of cell cycle arrest and apoptosis. In particular, an important role of p53 in the pathogenesis of vascular diseases is suggested by decreased p53 levels in human restenotic (22) and atherosclerotic lesions (23). The importance of p53 is also confirmed in various animal models. Adenovirus-mediated gene transfer of p53 to rat carotid arteries inhibited neointimal formation following balloon injury (24), whereas targeted deletion of p53 led to larger intimal lesions in a mouse vein graft model (25).

The mitogen-activated protein kinase (MAPK) p38 has been shown to be activated by cellular stress, UV light radiation, growth factor withdrawal, and pro-inflammatory cytokines (26–29). Upon activation, p38 phosphorylates various transcription factors but, of particular note, has been demonstrated to phosphorylate the tumor suppressor p53 (30). p38 MAPK is also implicated in both pro-apoptotic and anti-apoptotic signaling pathways (12, 31) However, its activity is likely cell-type-specific, with most studies focusing predominantly on inflammatory cells (32).

In this report we scrutinized the precise molecular mechanism of PKCδ-induced vascular SMC apoptosis and provide evidence that PKCδ activation leads to accumulation/modification of p53, which is essential for the induction of apoptosis. Moreover, the role of p38 as an intermediate in PKCδ-induced p53 accumulation and apoptosis has also been demonstrated. In total, these studies provide an explicit link...
between PKCδ, p38 activation, and p53 modulation in the process of SMC apoptosis. These findings have implications for the development of improved approaches for the prevention and management of restenosis.

EXPERIMENTAL PROCEDURES

General Materials—Phorbol 12-myristate 13-acetate (PMA) was purchased from Biomol (Plymouth Meeting, Pennsylvania), and dimethyl sulfoxide (Me₂SO), rottlerin, SR20358, and H₂O₂, along with other chemicals not specified, were purchased from Sigma. Dulbecco’s modified Eagle’s medium and cell culture reagents were from Invitrogen.

Antibodies—The rabbit polyclonal antibody to PKCδ and the mouse monoclonal antibody to β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, California). Polyclonal rabbit antibodies to cleaved caspase-3 and phospho-p38 were obtained from Cell Signaling Technology Inc. (Beverly, Massachusetts). Biotinylated p53 antibody and antibody to phosphorylated p53 were obtained from R&D Systems (Minneapolis, Minnesota).

SMC Culture—Rat aortic A10 SMCs, obtained from the American Tissue Culture Collection, were grown as recommended. Mouse aortic SMCs were isolated from the thoracic aorta of p53−/− male mice (33) (Jackson Laboratories, Bar Harbor, Maine) based on a protocol described by Clowes et al. (34) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C with 5% CO₂.

Construction of Adenoviral Vectors and Infection—A recombinant adenoviral vector was constructed to express PKCδ. Briefly, a DNA fragment containing the desired sequence was generated by PCR using the human cDNA as a template. Following DNA sequencing, the PCR product was then cloned into an E1- and E3-deficient adenoviral vector (pEasy). Adenoviruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation. A recombinant adenovirus encoding for the wild-type p53 protein was a generous gift from Enrico Ascher (24, 35).

Construction of Adenoviral Vectors and Infection—A recombinant adenoviral vector was constructed to express PKCδ. Briefly, a DNA fragment containing the desired sequence was generated by PCR using the human cDNA as a template. Following DNA sequencing, the PCR product was then cloned into an E1- and E3-deficient adenoviral vector (pEasy). Adenoviruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation. A recombinant adenovirus encoding for the wild-type p53 protein was a generous gift from Enrico Ascher (24, 35).

Construction of Adenoviral Vectors and Infection—A recombinant adenoviral vector was constructed to express PKCδ. Briefly, a DNA fragment containing the desired sequence was generated by PCR using the human cDNA as a template. Following DNA sequencing, the PCR product was then cloned into an E1- and E3-deficient adenoviral vector (pEasy). Adenoviruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation. A recombinant adenovirus encoding for the wild-type p53 protein was a generous gift from Enrico Ascher (24, 35).

Construction of Adenoviral Vectors and Infection—A recombinant adenoviral vector was constructed to express PKCδ. Briefly, a DNA fragment containing the desired sequence was generated by PCR using the human cDNA as a template. Following DNA sequencing, the PCR product was then cloned into an E1- and E3-deficient adenoviral vector (pEasy). Adenoviruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation. A recombinant adenovirus encoding for the wild-type p53 protein was a generous gift from Enrico Ascher (24, 35).

Construction of Adenoviral Vectors and Infection—A recombinant adenoviral vector was constructed to express PKCδ. Briefly, a DNA fragment containing the desired sequence was generated by PCR using the human cDNA as a template. Following DNA sequencing, the PCR product was then cloned into an E1- and E3-deficient adenoviral vector (pEasy). Adenoviruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation. A recombinant adenovirus encoding for the wild-type p53 protein was a generous gift from Enrico Ascher (24, 35).

RESULTS

PKCδ Induces SMC Apoptosis through p38 and p53—We began our studies by testing whether inhibition of PKCδ affects the ability of SMCs to undergo apoptosis. A10 SMCs were treated with the apoptotic stimulus H₂O₂ at a concentration of 200 μM for 6 h in the presence and absence of the PKCδ-specific inhibitor rottlerin. Similar to what has been reported previously in mouse vascular SMCs, A10 cells, a rat aortic SMC line, responded to H₂O₂ with a large increase in DNA fragmentation. Pre-treatment of A10 cells with rottlerin (1 μM for 1 h) diminished the induction of DNA fragmentation induced by H₂O₂ (Fig. 1A). Alternatively, we inhibited PKCδ expression by treating cells with an antisense oligonucleotide. Compared with the control oligo, PKCδ antisense oligo produced a significant reduction in the level of endogenous PKCδ (Fig. 1B). More importantly, the PKCδ antisense oligo-treated cells became resistant to H₂O₂ treatment (Fig. 1B). Taken together, these data confirmed that PKCδ is a necessary component of the apoptotic pathway in vascular SMCs.

Overexpression of PKCδ Induces Apoptosis and Accumulation of the Tumor Suppressor p53—We next evaluated whether overexpression of PKCδ would be sufficient to induce vascular SMC apoptosis. To this end, we employed an adenovirus encoding full-length wild-type PKCδ (AdPKCδ), which led to a marked increase in cellular levels of PKCδ (Fig. 2B). Additionally, AdPKCδ induced a small but significant elevation in the level of fragmented DNA and cleaved caspase-3 (Fig. 2A and B). To facilitate the activation of the ectopically expressed PKCδ, we treated A10 SMCs with 1–5 μM of PMA for 12 h. At these concentrations, PMA alone did not induce apoptosis as indicated by the lack of fragmented DNA as well as the absence of activated caspase-3. However, the PKCδ activator PMA, in combination with overexpression of PKCδ, resulted in an increase in DNA fragmentation by >3-fold (Fig. 2A) and cleaved caspase-3 by >300% (Fig. 2B). These data establish that overexpression of PKCδ is sufficient to induce SMC apoptosis. Because p53 has been implicated in SMC apoptosis, we investigated whether the overexpression of PKCδ and its dramatic increase in SMC apoptosis were associated with an induction of p53. As shown in Fig. 2B, overexpression of PKCδ in A10 SMCs significantly increased p53 levels. In parallel to the induction of apoptosis, the ability of AdPKCδ to induce p53 expression was further enhanced by PMA (Fig. 2B). Next, we tested
whether the PKCδ-induced p53 accumulation leads to enhanced p53-dependent gene transcription by using a luciferase reporter gene containing a p53-specific enhancer element (40, 41). Co-transfection of a PKCδ expression vector significantly increased p53 reporter activity. More importantly, the addition of the PKC activator PMA facilitated the effect of PKCδ on the p53 reporter, which is consistent with the ability of PKCδ to induce p53 expression and SMC apoptosis (Fig. 2C).

p53 Is Necessary for PKCδ-induced Apoptosis—To confirm the significance of p53 in PKCδ-induced apoptosis, we designed a specific p53 small interference RNA (siRNA) to block p53 translation. 72 h following the administration of this p53 siRNA (50 nM), levels of p53 in A10 SMCs were decreased by 44 ± 2.2% (Fig. 3A). Next, we examined whether the p53 siRNA affects apoptosis. 48 h following infection with AdPKCδ or AdNull, A10 cells were incubated with p53 siRNA (50 nM for 72 h) prior to PMA treatment (1 μM for 12 h). Cell apoptosis was then assessed using ELISA for DNA fragmentation and Western blot analysis for cleaved caspase-3. Inhibition of p53 with the specific siRNA led to a significant decrease in both PKCδ-induced cleaved caspase-3 (Fig. 3A) and DNA fragmentation (Fig. 3B), suggesting that p53 is necessary for PKCδ-induced SMC apoptosis. To confirm these findings using siRNA, we isolated SMCs from the thoracic aorta of p53 null mice and tested their ability to undergo apoptosis following overexpression of PKCδ and activation with PMA. Interestingly, AdPKCδ failed to induce apoptosis in p53 null SMCs (Fig. 3C). Next, we attempted to rescue apoptosis by restoring p53 expression using an adenovirus encoding wild type p53 (35). Overexpression of p53 alone did not induce apoptosis, which is consistent with reports in HCT116 colon carcinoma cells (42). However, the expression of p53 restored completely the ability of PKCδ to induce apoptosis (Fig. 3C). These results provide further confirmation of the requirement of p53 for PKCδ-induced apoptosis of vascular SMCs.

PKCδ Increases p53 Transcription—We next explored the mechanism by which PKCδ regulates p53. We began by examining the effect of PKCδ overexpression on the level of p53 mRNA. A10 SMCs were infected with AdNull or AdPKδ and then treated with the PKC activator PMA (1 μM for 6 h). Total RNA was isolated from control or PKCδ/PMA-treated cells, and p53 was quantified using TaqMan real time PCR (Fig. 3D). The results show that PKCδ induced a significant increase in p53 mRNA levels (Fig. 3D). This stimulation was evident at 8 h and was consistent with the p53 protein levels (Fig. 2B). These results demonstrate that PKCδ transcriptionally activates p53, providing a mechanism by which PKCδ induces apoptosis.

PKCδ Induces SMC Apoptosis through p38 and p53

FIGURE 1. PKCδ is necessary for SMC apoptosis. A, A10 cells were treated with H2O2 (200 μM) for 6 h. Where indicated, cells were pretreated with rottlerin (1 μM) or control solvent (Me2SO) for 1 h prior to the addition of H2O2. Apoptosis was quantified using ELISA-measured DNA fragmentation as described under “Experimental Procedures.” B, A10 SMCs were evaluated following incubation with a PKCδ-specific antisense oligonucleotide (10 nM) for 48 h prior to H2O2 treatment (200 μM for 6 h). DNA fragmentation was quantified via ELISA. *, p < 0.05 as compared with non-treated control; n = 3.

FIGURE 2. Overexpression of PKCδ induces expression of the tumor suppressor p53 and SMC apoptosis. A, A10 SMCs were infected with an adenovirus containing wild type PKCδ (AdPKδ) or an empty viral vector (AdNull). Following infection, cells were treated with indicated concentrations of PMA or equal amounts of solvent (Me2SO) for 12 h. Apoptosis was determined by ELISA-measured DNA fragmentation. B, A10 cells were treated exactly as described for panel A. Cell lysate was analyzed with anti-PKCδ, p53, and cleaved caspase-3 antibody. C, A10 cells that were transiently transfected with a luciferase reporter gene under the control of p53-specific enhancer elements and either a PKCδ expression or control plasmid. Following infection, cells were treated with PMA (1 μM for 12 h). Reporter activity is expressed as a ratio of firefly luciferase to renilla luciferase. *, p < 0.05; **, p < 0.01 as compared with non-PMA treated control; n = 3.
time reverse transcription PCR analysis. As shown in Fig. 4A, overexpressed PKCδ elicited a significant increase in the level of p53 mRNA. To determine whether the increase in p53 mRNA is secondary to increased promoter activity, we employed a luciferase construct containing the proximal portion of the human p53 promoter (37). We co-transfected A10 cells with a PKCδ expression or control vector and the luciferase reporter. This experiment demonstrated an increase in p53 promoter activity in response to PKCδ overexpression (Fig. 4B), indicating that PKCδ regulates p53 transcription by up-regulating promoter activity in vascular SMCs.

Overexpression of PKCδ Results in the Phosphorylation of p53—Because phosphorylation is an important element of p53 regulation, we next investigated the possibility that PKCδ may affect p53 through phosphorylation. To assess PKCδ-induced p53 phosphorylation, we utilized a specific antibody to p53 phosphorylated on serine residue 46, shown by others to provide p53 with greater affinity to promoters of

**FIGURE 3.** p53 is necessary for PKCδ-induced SMC apoptosis. A and B, A10 SMCs were infected with AdPKCδ. Cells were then incubated with p53 siRNA or scrambled control siRNA (50 nM) for 72 h and then with PMA (1 μM for 12 h). p53 inhibition was confirmed through Western blot analysis of cell lysate (A). Apoptosis was assessed by measuring levels of cleaved caspase-3 via immunoblot (A) and ELISA-measured DNA fragmentation (B). C, SMCs were harvested from the thoracic aorta of p53−/− male mice. 24 h following seeding at equal densities in 10% fetal bovine serum media, mouse aortic SMCs were infected with equal quantities of AdNull, AdPKCδ, or adenovirus (Ad) p53 (30,000 total viral particles per cell). Following PMA treatment (1 μM for 12 h), apoptosis was evaluated though ELISA-measured DNA fragmentation. *, p < 0.05 as compared with non-treated control; n = 3.
PKCδ Induces SMC Apoptosis through p38 and p53

FIGURE 4. PKCδ increases p53 transcription. A, A10 SMCs, infected with a PKCδ adenovirus (AdPKCδ) or empty viral vector (AdNull), were stimulated for 6 h with 1 μM of PMA. The level of p53 mRNA was determined with real time reverse transcription PCR. B, A10 cells were transfected with a p53 luciferase reporter and either PKCδ or a control vector. Following transfection, cells were stimulated for 12 h with 1 μM PMA. Reporter activity is expressed as a ratio of firefly luciferase to renilla luciferase. *, p < 0.05 as compared with non-treated control; n = 3.

apoptosis-related genes (43). PKCδ substantially increased the level of phospho-p53 (at serine 46), which was barely detectable in cells infected with the empty viral vector (Fig. 5A). To further confirm the role of PKCδ-induced p53 phosphorylation in apoptosis, we evaluated the phosphorylation status of p53 in A10 cells treated with H2O2, a stimulus for both apoptosis and p53 phosphorylation (44). Indeed, Western blot analysis of A10 cell lysates demonstrated a significant increase in p53 phosphorylation at serine 46 following treatment with H2O2 (200 μM for 4 h). Importantly, pre-incubation with the PKCδ chemical inhibitor rottlerin (1 μM for 1 h) dramatically diminished the ability of H2O2 to induce p53 phosphorylation (Fig. 5B). These data demonstrate that PKCδ is responsible, directly or indirectly, for p53 phosphorylation. Lastly, we searched for the mechanism that enables the interaction between PKCδ and p53 by examining the potential physical association between the two proteins. Lysates from A10 SMCs infected with AdPKCδ were immunoprecipitated for p53 followed by Western blotting for PKCδ. As a negative control, the same cell lysate was immunoprecipitated with normal rabbit IgG. We found a prominent p53 band in the PKCδ immunoprecipitate. In the converse experiment, PKCδ was detected in the p53 immunocomplex (Fig. 5C). These findings suggest that PKCδ is physically associated with p53 in conditions favoring apoptosis.

p38 MAPK Is Necessary for PKCδ-induced Up-regulation of p53—Next, we explored the molecular mechanisms underlying the p53 induction by evaluating the stress-activated kinase p38 in response to PKCδ overexpression. Overexpression of PKCδ in A10 SMCs activated p38 as demonstrated by the marked increase in p38 phosphorylation (Fig. 6A). To investigate a possible role of p38 in PKCδ-induced p53 expression, we inhibited p38 using the p38 chemical inhibitor SB20358 (20 μM for 1 h) in PKCδ-overexpressing cells. We found that p38 inhibition resulted in a large decrease in the ability of PKCδ to up-regulate p53 protein levels (Fig. 6B). Next, we tested the effect of SB20358 on p53 promoter activity. As shown above, p53 promoter activity was increased in response to PKCδ overexpression. However, inhibition of p38 by SB20358 diminished the ability of PKCδ to up-regulate p53 transcription (Fig. 6C). These data suggest that p38 is the intermediate responsible for the induction of p53 transcription in PKCδ-overexpressing SMCs.

FIGURE 5. Overexpression of PKCδ results in p53 phosphorylation. A, A10 SMCs infected with AdPKCδ or AdNull were stimulated for 12 h with 1 μM of PMA. Cell lysates were examined via Western blotting for total or phosphorylated p53. B, A10 cells were treated with H2O2 (200 μM for 4 h) and pre-incubated with the PKCδ chemical inhibitor rottlerin (1 μM for 1 h) or control solvent (Me2SO) as indicated. Western blot analysis was performed with an antibody specific for phospho-p53 (serine 46). C, A10 SMCs were infected with AdPKCδ and stimulated PMA (1 μM for 12 h). Cell lysates were immunoprecipitated with an anti-p53 or anti-PKCδ antibody. As a negative control, cell lysates were also immunoprecipitated with normal IgG. Immunoprecipitate was analyzed for PKCδ or p53 via Western blot (WB) analysis.

p38 MAPK Is Not Necessary for PKCδ-induced p53 Phosphorylation—Because investigations in non-vascular cell types have demonstrated that p38 MAPK is a potential p53 serine 46 kinase and that p53-mediated apoptosis is dependent on this event, we explored whether these findings apply to SMCs. Chemical inhibition of p38 failed to block PKCδ-induced p53 phosphorylation but did decrease total p53 protein levels as demonstrated above (Fig. 7A). Despite the lack of influence of p38 MAPK on serine 46 phosphorylation, we found that both proteins were co-associated with PKCδ by a co-immunoprecipitation assay (Fig. 7B).

p38 MAPK Is Necessary for PKCδ-induced SMC Apoptosis—After establishing a potential direct interaction between PKCδ and p38 and the necessity of p38 for PKCδ-induced up-regulation of p53, we investigated the role of p38 MAPK in SMC apoptosis using the p38 inhibitor SB20358. Interestingly, a treatment with this inhibitor (20 μM for 1 h) prior to PMA activation (1 μM for 12 h) decreased apoptosis by >50% as quantified by both cleaved caspase-3 (Fig. 8A) and DNA fragmentation...
Thus, p38 MAPK contributes in part to PKCδ-induced SMC apoptosis.

DISCUSSION

PKCδ, a member of the novel PKC subfamily, can be pro-apoptotic or anti-apoptotic depending on cell types and stimuli. In agreement with a previous report (21), our results support the notion that PKCδ is pro-apoptotic in vascular SMCs. Because molecular activation of PKCδ alone was sufficient to result in caspase-3 activation and DNA fragmentation, we speculate that activation of PKCδ is an early event leading to the onset of programmed cell death in SMCs.

The molecular mechanisms linking PKCδ to the induction of apoptosis have been explored to some extent in non-smooth muscle cells. Several studies suggest that the presence of a positive regulatory loop between PKCδ and caspase-3; however, exactly how PKCδ might stimulate caspase-3 remains unclear (45, 46). Another important target of PKCδ is the mitochondria. It was demonstrated in HeLa cells (47) and keratinocytes (48) that overexpression and activation of PKCδ leads to a reduction in mitochondrial membrane potential and release of cytochrome c, which subsequently leads to activation of caspases and apoptosis. Additionally, several nuclear proteins, including DNA-dependent protein kinase (49), p73β (50), and lamin B (51) have been identified as PKCδ targets/substrates. Activated PKCδ associates with and phosphorylates these proteins; such interactions, at least in part, contribute to apoptosis. In the current study, we present evidence that the tumor suppressor p53 is a necessary mediator of PKCδ-induced apoptosis in

FIGURE 6. p38 MAPK is necessary for PKCδ-induced up-regulation of p53 expression. A, A10 SMCs were infected with AdPKCδ or AdNull and stimulated with PMA (1 μM for 12 h). Cell lysate was examined via Western blotting for total and phosphorylated p38 MAPK. B, A10 cells infected with AdPKCδ underwent pretreatment with control solvent (Me2SO) or the p38 MAPK inhibitor SB20358 (20 μM for 1 h) prior to stimulation with PMA (1 μM for 12 h). Total p53 protein levels were assessed via Western blot. C, A10 cells were co-transfected with a PKCδ expression or control vector and the luciferase construct containing the p53 promoter. SB20358 (20 μM for 1 h) was used to inhibit p38 activity prior to the addition of PMA (1 μM for 12 h); p53 reporter activity was expressed as a ratio of firefly luciferase to renilla luciferase. *, p < 0.05 as compared with non-treated control.

FIGURE 7. p38 co-associates with both PKCδ and p53 but is not necessary for PKCδ-induced p53 phosphorylation. A, A10 SMCs, infected with AdPKCδ or AdNull, were preincubated with the p38 MAPK inhibitor SB20358 (20 μM for 1 h) prior to stimulation with PMA (1 μM for 12 h). Cell lysate was examined via Western blotting using a phospho-p53 antibody specific for serine residue 46. B, A10 SMCs were infected with AdPKCδ and stimulated for PMA (1 μM for 12 h). Cell lysates were immunoprecipitated with an anti-p38 antibody. As a negative control, cell lysates were also immunoprecipitated with normal IgG. The immunoprecipitate was analyzed for PKCδ and p53 via Western blot (WB) analysis.

FIGURE 8. p38 MAPK is necessary for PKCδ-induced SMC apoptosis. A, A10 SMCs, infected with AdPKCδ, were preincubated with the p38 MAPK inhibitor SB20358 (20 μM for 1 h) prior to stimulation with PMA (1 μM for 12 h). Apoptosis was assessed by measuring levels of cleaved caspase-3 via immunoblot (A) and ELISA-measured DNA fragmentation (B). *, p < 0.05 as compared with non-treated control.
PKCδ Induces SMC Apoptosis through p38 and p53

vascular SMCs. We have shown that activation of PKCδ led to accumulation as well as phosphorylation of p53 in SMCs; this induction correlated with SMC apoptosis. Moreover, blocking p53 induction with siRNA prevented apoptosis. Finally, targeted gene deletion of p53 prevented PKCδ-induced apoptosis, whereas restoring p53 expression through adenovirus-mediated p53 gene transfer rescued the ability of PKCδ to induce apoptosis. To our knowledge, this is the first demonstration of the direct involvement of p53 in the regulation of SMC apoptosis by PKCδ.

Experimental evidence is now provided that PKCδ regulates p53 at both transcriptional and post-translational levels, apparently mediated by separate signaling mechanisms. Specifically, the transcriptional regulation requires p38 MAPK, whereas the post-translational modification, at least for Ser-46, does not require MAPK. The importance of post-translational regulation of p53 by PKCδ is demonstrated by our observation that ectopic expression of p53 alone was insufficient to induce apoptosis. Only when co-expressed with PKCδ was p53 able to restore apoptosis of p53 null cells, presumably through a PKCδ-dependent phosphorylation. It was surprising that the p38 MAPK-specific inhibitor, SB20358, significantly inhibited PKCδ-induced accumulation of p53 but did not affect p53 phosphorylation at Ser-46, because direct interaction between p53 and p38 MAPK (52) has been suggested previously. Indeed, we showed that all three proteins, p53, p38 and PKCδ, co-associated in the immunoprecipitation complex isolated from SMC lysates. Our attempts to evaluate other serine residues within the p53 molecule are currently not possible due to a lack of specific phosphoantibodies. Therefore, it remains to be determined whether PKCδ and p38 stimulate p53 phosphorylation at additional residues. With respect to Ser-46, our data suggest that it is p38-independent.

The potential role of PKCδ in the regulation of p53 accumulation has been suggested previously. Using the selective PKCδ inhibitor rottlerin, Niwa et al. (44) demonstrated that the inhibition of PKCδ decreases H2O2-induced p53 accumulation in bovine endothelial cells. In NIH3T3 cells, Lee et al. (53) observed an increase in the p53 level by overexpressing PKCζ, whereas Abbas et al. (54) demonstrated a suppression of p53 basal expression by inhibiting PKCζ in ML-1 cells (acute myeloid leukemia cells). We have now demonstrated that in vascular SMCs the overexpression of PKCδ increased the accumulation of both the p53 protein and mRNA. In contrast, Johnson et al. (55) found that the PKCδ inhibitor rottlerin increases p53 levels in cisplatin-treated HeLa cells, whereas the PKC activator phorbol 12,13-dibutyrate attenuates p53 levels in the same cell line. Therefore, it is likely that PKCδ, paralleling its dual functions in apoptosis, plays multiple roles in regulating p53 expression, which is dependent upon the cell type and stimuli employed.

Our findings that PMA alone did not induce apoptosis in control or AdNull-infected SMCs is seemingly surprising, because these cells express endogenous PKCδ, and PMA activates PKCδ. The lack of cell death associated with PMA treatment may be related to the effect of PMA on other PKC isotypes. We have shown previously that SMC express at least eight isotypes of PKC, among which six can be activated by PMA (56). Some of these PKC-sensitive PKC isoforms, such as PKCζ, are pro-apoptotic, whereas others, such as PKCα, have been demonstrated to be anti-apoptotic in non-SMCs (57–59). Our data show that overexpressing PKCζ shifts the PMA response in favor of apoptosis. However, the outcome of simultaneously activating multiple PKC isotypes, as may occur in control SMCs treated with PMA, appears to result in no net effect on cell death.

The present study highlights another interesting finding that PKCδ stimulates p53 gene expression through p38 MAPK. We showed that inhibition of p38 completely eliminated PKCδ-stimulated accumulation of p53 protein and mRNA. Moreover, analyses using a p53 promoter reporter demonstrated that PKCδ up-regulated p53 promoter activity, also in a p38-dependent manner. Several transcription factors have been identified to bind and regulate the murine p53 promoter, including NFκB (60). Interestingly, Kim et al. (61) showed in articular chondrocytes that NO-induced activation of p38 up-regulates p53 expression through NFκB. Moreover, the NFκB pathway has been shown to be activated by PKCδ in several cell types (62–64). Future studies are mandatory to directly test the role of NFκB in PKCδ-induced p38-dependent transcriptional regulation of p53.

In summary, our results demonstrate that PKCδ plays a pivotal role in the signal transduction pathway leading to vascular SMC apoptosis. The p38-dependent-accumulation and independent-phosphorylation of p53 by PKCδ contributes, at least in part, to SMC apoptosis. Given the critical role of apoptosis in interimal hyperplasia, it is possible that enhancement of PKCδ activity at different stages after vascular intervention may provide a new strategy for the prevention and treatment of restenosis.

Acknowledgments—We thank Dr. E. Ascher (Maimonides Medical Center, Brooklyn, NY) for the recombinant adenovirus p53 vector and Dr. D. Resiman (University of Wisconsin, Madison, WI) for the p53 luciferase construct. We also thank Dr. N. Heckatte at The Gene Therapy Core Facility, Weill Cornell Medical College for assistance with adenovirus preparation and Sophia Chu for technical assistance.

REFERENCES

1. Kerr, J. F. R., and Harmon, B. V. (1991) in Apoptosis: The Molecular Basis of Cell Death (Torrei, L. D., and Cope, F. O., eds.) Vol. 3, pp. 5–29, Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY.

2. Wyllie, A. (1992) Cancer Metastasis. Rev. 11, 95–103

3. Bauriedel, G., Schluckebier, S., Hutter, R., Welsch, U., Kandolf, R., Luderitz, B., and Prescott, M. F. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1132–1139

4. Han, D., Haudenschild, C., Hong, M., Tinkle, B., Leon, M., and Liau, G. (1999) Am. J. Pathol. 147, 267–277

5. Bochaton-Piallat, M., Gabbiani, F., Redard, M., Desnosoliere, A., and Gabbiano, G. (1995) Am. J. Pathol. 146, 1059–1069

6. Ross, R. (1993) Nature 362, 801–809

7. Xu, Q. (2000) Trends Cardiovasc. Med. 10, 35–41

8. Geng, Y., and Libby, P. (1995) Am. J. Pathol. 147, 251–266

9. Isner, J. M., Kearney, M., Bortman, S., and Passeri, J. (1995) Circulation 91, 2701–2711

10. Mayr, U., Mayr, M., Li, C., Wernig, F., Dietrich, H., Hu, Y., and Xu, Q. (2002) Circ. Res. 90, 1505–1512

11. Newby, A. C., and George, S. J. (1996) Curr. Opin. Cardiol. 11, 574–582

12. Kockx, M. M. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1519–1522

13. Franklin, R. A., and McCubre, J. A. (2000) Leukemia 14, 2019–2034

14. Cross, T. G., Scheel-Toellner, D., Hentizque, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) Exp. Cell Res. 256, 34–41

15. Kromer, G. (1997) Nat. Med. 3, 614–620

16. Strasser, A., Connor, L., and Dixit, V. M. (2000) Annu. Rev. Biochem. 69, 217–245

17. Hug, H., and Sarre, T. (1993) Biochem. J. 15, 329–343

18. Fukushima, S., Nishizawa, Y., Hosoi, M., Koyama, H., Yamakawa, K., Ohno, S., and Morii, H. (1997) J. Biol. Chem. 272, 13816–13822

19. Li, W., Jiang, Y.-X., Zhang, J., Soon, L., Flechner, L., Kapoor, V., Pierce, J. H., and Wang, L.-H. (1998) Mol. Cell. Biol. 18, 5888–5898

20. Leitges, M., Mayr, M., Braun, U., Mayr, U., Li, C., Pfister, G., Ghaffari-Tabrizi, N., Baier, G., Hu, Y., and Xu, Q. (2001) J. Clin. Investig. 108, 1505–1512

21. Yoneimitsu, Y., Kaneda, Y., Tanaka, N., Nakashima, Y., Komori, K., Sugimaki, K., and Sueishi, K. (1998) Circ. Res. 82, 147–156

22. George, S. J., Angelini, G. D., Capogrossi, M. C., and Baker, A. H. (2001) Gene Ther. 8, 668–676

23. Scheinman, M., Ascher, E., Levi, G. S., Hingorani, A., Shiraizian, D., and Seth, P. (1999) J. Vasc. Surg. 29, 360–369

24. Mayr, U., Mayr, M., Li, C., Wernig, F., Dietrich, H., Hu, Y., and Xu, Q. (2002) Circ. Res. 100, 967–973
Protein Kinase C Delta Induces Apoptosis of Vascular Smooth Muscle Cells through Induction of the Tumor Suppressor p53 by Both p38-dependent and p38-independent Mechanisms
Evan J. Ryer, Kenji Sakakibara, Chunjie Wang, Devanand Sarkar, Paul B. Fisher, Peter L. Faries, K. Craig Kent and Bo Liu

J. Biol. Chem. 2005, 280:35310-35317. doi: 10.1074/jbc.M507187200 originally published online August 23, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M507187200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 23 of which can be accessed free at http://www.jbc.org/content/280/42/35310.full.html#ref-list-1