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Different Signaling Responses to Anti-Proliferative Agents in Human Aortic and Venous Smooth Muscle Cells

Ray M. Lee,[1,2]* Takahisa Masaki,[1] Hung-Sheng Yang,[2] Jihua Liu,[2] Jun Chen,[2] Li Li,[1] Donald K. Blumenthal,[3] and Alfred K. Cheung[1,4]**

[1]Department of Internal Medicine, University of Utah, Salt Lake City, Utah
[2]Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah
[3]Department of Pharmacology & Toxicology, University of Utah, Salt Lake City, Utah
[4]Veterans Affairs Salt Lake City Healthcare System, Salt Lake City, Utah

Abstract Proliferation of smooth muscle cells (SMCs) contributes to the stenosis of coronary arteries and vascular grafts. Local delivery of anti-proliferative drugs can prevent vascular stenosis. To understand the cellular responses to anti-proliferative agents, we investigated the signaling events in cultured human aortic SMCs (ASMCs), saphenous venous SMCs (VSMCs), and dermal fibroblasts (DFs) in response to paclitaxel or etoposide. Cellular mitochondrial and proliferative activities were examined with the methylthiazoletetrazolium (MTT) dye reduction and the bromodeoxyuridine (BrdU) incorporation assay, respectively. Cell proliferation was almost completely suppressed by paclitaxel or etoposide, but apoptosis was achieved in only about 50% of cells at the highest drug concentrations, suggesting the presence of compensatory mechanisms to prevent apoptosis. Examination of three important signaling pathways revealed significant differences between ASMCs, VSMCs, and DFs. Treatment with either paclitaxel or etoposide caused a transient phosphorylation/activation of p42 MAPK in ASMCs and DFs, but had no effect on phospho-p42/44 MAPK in VSMCs. High-dose etoposide enhanced p38 MAPK activation in ASMCs, but not in VSMCs. The p38 inhibitor, PD169316, partially inhibited etoposide-induced ASMC apoptosis, but induced apoptosis in VSMCs. The effects of etoposide and paclitaxel on Akt also differed between ASMCs and VSMCs. These observations indicate that ASMCs and VSMCs differ in the response of signaling pathways to anti-proliferative agents. In ASMCs, p42/44 MAPK appears to serve a pro-survival role, whereas p38 MAPK is a pro-apoptotic regulator. In contrast, p38 MAPK is an important pro-survival regulator in VSMCs and p42/44 MAPK appears to play a minor role in responding to anti-proliferative drugs. J. Cell. Biochem. 99: 835–844, 2006.
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Proliferation of smooth muscle cells (SMCs) leads to stenosis of coronary arteries and arteriovenous grafts used for vascular access in chronic hemodialysis patients [Swedberg et al., 1989; Linde and Strauss, 2001; Roy-Chaudhury et al., 2001]. Inhibition of proliferation and induction of apoptosis in vascular SMCs are strategies to alleviate stenosis. However, upon treatment with these anti-proliferative agents, cells frequently develop compensatory responses to maintain their survival [Clark et al., 2002]. Understanding the signaling events that mediate these compensatory responses will facilitate the development of new strategies to enhance apoptosis induced by currently available pharmacologic agents.

Many cell survival and proliferation events are regulated by protein kinases. One important kinase cascade is the Ras/Raf-1/MEK/(p42/44) MAPK pathway, which also regulates the

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*Correspondence to: Ray M. Lee, MD, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298. E-mail: rlee5@vcu.edu

**Correspondence to: Alfred K. Cheung, MD, Dialysis Program, University of Utah, 85 North Medical Drive East, Salt Lake City, UT 84112.
E-mail: alfred.cheung@hsc.utah.edu

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action of many anti-proliferative agents [Chen et al., 2001; Pearson et al., 2001]. Members of this kinase cascade are ubiquitously expressed and may be responsible for certain apoptotic checkpoints through phosphorylation of various important downstream substrates. Activation of the p42/44 MAPK pathway has been shown to be associated with inhibition of caspase activation and protection of cells from apoptosis [Xia et al., 1995; Le Gall et al., 2000; von Gise et al., 2001]. One substrate of p42/44 MAPK is caspase 9. Phosphorylation of caspase 9 by p42/44 MAPK prevents the activation of caspase 9 and the downstream caspase 3 [Allan et al., 2003]. Another substrate of p42/44 MAPK is Bcl-2, and phosphorylation of Bcl-2 at Ser-70 leads to stabilization of the Bcl-2/Bax complex and suppression of apoptosis in IL-3-dependent myeloid cells [Deng et al., 2000]. Another member of the MAPK family closely involved in apoptosis is p38 MAPK, which has an opposing effect to p42/44 MAPK on apoptosis [Xia et al., 1995]. Blocking p38 MAPK activity with the small molecule inhibitor, PD169316, prevents apoptosis induced by the withdrawal of nerve growth factor (NGF) from neurons [Kummer et al., 1997]. PD169316 also protects endothelial cells from apoptosis induced by γ-irradiation [Kumar et al., 2004].

Another important protein kinase cascade related to cell survival is the phosphatidylinositol-3 kinase (PI3K)/PTEN/Akt pathway [Datta et al., 1999]. Activation of growth factor receptor tyrosine kinases leads to activation of PI3K, which generates phosphatidylinositol-3,4,5-triphosphate (PIP3) that activates Akt. The tumor suppressor gene PTEN encodes a PIP3 phosphatase that suppresses Akt activation and serves as the brake for this pathway [Thompson and Thompson, 2004; Kim et al., 2005]. A number of downstream effectors of Akt involved in regulation of apoptosis are known, including Bad [Datta et al., 1997], caspase 9 [Cardone et al., 1998], Forkhead transcription factors [Brunet et al., 2001], and the cell cycle regulator, p27 [Shin et al., 2002]. The PI3K/PTEN/Akt pathway is frequently altered in many human cancers and overexpression of Akt can contribute to tumor transformation and resistance to chemotherapy [Thompson and Thompson, 2004; Kim et al., 2005].

In this study, we investigated the signaling events in aortic SMCs (ASMCs), saphenous venous SMCs (VSMCs), and dermal fibroblasts (DFs) after treatment with either of two anti-proliferative agents, paclitaxel and etoposide. Paclitaxel is a microtubule-interfering agent and has been shown to inhibit ASMC proliferation and migration [Axel et al., 1997; Kim et al., 2004]. It is now commonly used in drug-eluting stents to prevent post-angioplasty coronary artery stenosis [Park et al., 2003; Ajani and Waksman, 2004; Stone et al., 2004; Waugh and Wagstaff, 2004]. Recent studies in animal models have also suggested that the perivascular [Masaki et al., 2004] or intraluminal sustained-delivery of paclitaxel by stents is effective in preventing stenosis of arteriovenous hemodialysis grafts. Etoposide is a topoisomerase II inhibitor commonly used to suppress cell proliferation and induce apoptosis partly through a p53-dependent pathway [de la Llera-Moya et al., 1992; Scott et al., 2002]. The goal of this study is to understand how ASMCs and VSMCs respond differently to cytotoxic agents with regard to these two important kinase pathways and the compensatory mechanisms that could lead to resistance to apoptosis. DFs are also important to consider in perivascular drug delivery, since the drug is deposited at a location that would allow interaction with these cells in subcutaneous tissues. Understanding the various cell signaling events in vascular SMCs and fibroblasts could lead to the development of novel strategies to inhibit vascular stenosis more effectively.

MATERIALS AND METHODS

Materials

Human ASMCs, saphenous VSMCs, and DFs were purchased from Cambrex (Chicago, IL). Paclitaxel and etoposide were purchased from Sigma Chemical Inc. (St. Louis, MO), and PD98059 and PD169316 were from Calbiochem (San Diego, CA). Each drug was dissolved in dimethyl sulfoxide (DMSO) and diluted immediately before adding to the tissue culture media. Methylthiazoletetrazolium (MTT) was purchased from Calbiochem. The 5-bromo-2′-deoxyuridine (BrdU)-labeled DNA colorimetric ELISA kit was purchased from Amersham (Piscataway, NJ). The Akt, phospho-Akt, p42/44 MAPK, phospho-p42/44 MAPK, p38 MAPK, and phospho-p38 MAPK antibodies for the Western blotting experiments were all purchased from Cell Signaling Inc. (Cambridge, MA). The β-actin antibody was from Sigma Chemical Inc.

Cell Culture

ASMCs and VSMCs were cultured at 37°C in a humidified 5% CO_2 incubator in 10% fetal bovine serum (FBS) and SMC Growth Medium-2 Bullet Kit (Cambrex) supplemented with 5 μg/ml insulin, 0.5 ng/ml human recombinant epidermal growth factor, 2 ng/ml human recombinant fibroblast growth factor, 50 μg/ml gentamycin, and 50 ng/ml amphotericin-B. DFs were grown in 10% FBS and Fibroblast Growth Medium-2 (FGM-2, Cambrex) supplemented with 2 ng/ml human recombinant fibroblast growth factor, 5 μg/ml insulin, 50 μg/ml gentamycin, and 50 ng/ml amphotericin-B. For all experiments, SMCs and DFs at passages six–eight were used.

Proliferation and Apoptosis Assays

The cultured SMCs and DFs were seeded at a density of 1×10^4 cells per well in 96-well tissue culture plates and incubated at 37°C in 5% CO_2. After 48 h, the culture medium was replaced with serum-free medium to induce quiescence. After another 48 h, the medium was replaced with 100 μl of fresh medium containing 20% FBS and various concentrations of paclitaxel or etoposide. Drugs were dissolved in DMSO, and the same concentrations of DMSO (0.1%) in the absence of drugs served as negative (vehicle) controls. After addition of drug or DMSO alone, cells were cultured for an additional 48 h. Cell proliferation was assessed by the BrdU incorporation assay, which was performed using a commercial ELISA kit according to the manufacturer's instructions. The apoptosis assay was performed using the MTT assay. In brief, after incubation with the drug or vehicle for 48 h, cells were incubated with the MTT labeling solution (0.5 mg/ml) at 37°C for another 4 h. The cells were then solubilized in 100 μl of 0.1 N HCl and isopropyl alcohol and shaken for 30 s on a plate rotator. The absorbance at 570 nm was then measured using a microtiter plate reader with the reference wavelength at 630 nm.

Statistical Analysis

The absorbance values in the proliferation and apoptosis assays were determined in triplicate. The averaged values were normalized against the mean control value from the same experiment using the following formula: (sample absorbance/control absorbance) $\times$ 100%. All experiments were performed at least three times on three separate days for each drug concentration to calculate the mean $\pm$ SD.

Western Blotting

After incubation with drugs, cells were harvested at different time points using a lysis buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and sonication to break cellular DNA. The lysates were centrifuged at 12,000*g* and the supernatants analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electrophoretic transfer to an Immobilon-P membrane (Millipore, Inc.) for Western blotting analysis.

RESULTS

Sensitivity of ASMCs and VSMCs to Paclitaxel and Etoposide

To determine the sensitivity of ASMCs, VSMCs, and DFs to paclitaxel and etoposide, we incubated cells with paclitaxel or etoposide for 48 h and the degree of apoptosis was evaluated by MTT assays. The concentrations of paclitaxel were 0.05, 0.1, 0.5, 1.0, and 10 μM and those of etoposide were 5, 10, 20, 50, and 100 μM; these concentration ranges were based on dose-response analyses done previously in this laboratory (data not shown). All three cell types, ASMCs, VSMCs, and DFs, appeared to undergo similar degrees of apoptosis, with the absorbance decreased to 50% in the MTT assay at the highest drug concentrations ($P < 0.05$ compared with the control; Fig. 1a,b). The difference in the extent of decrease in response to either paclitaxel or etoposide was not statistically significant between ASMCs and VSMCs. Only DFs treated with 10 μM paclitaxel showed statistical significance compared with ASMCs or VSMCs (Fig. 1a). Neither drug exhibited a clear dose-response in the MTT assay, which may be due to the possibility that cell death might also involve other pathways unrelated to disruption of mitochondrial potential. Cell proliferation was then investigated using BrdU incorporation. Despite the modest degree of apoptosis measured by MTT assays, all three cell types lost the ability to incorporate BrdU even at the lowest concentrations of paclitaxel and etoposide, indicating that proliferation was almost completely suppressed when these drugs reached a low threshold concentration (Fig. 1c,d).

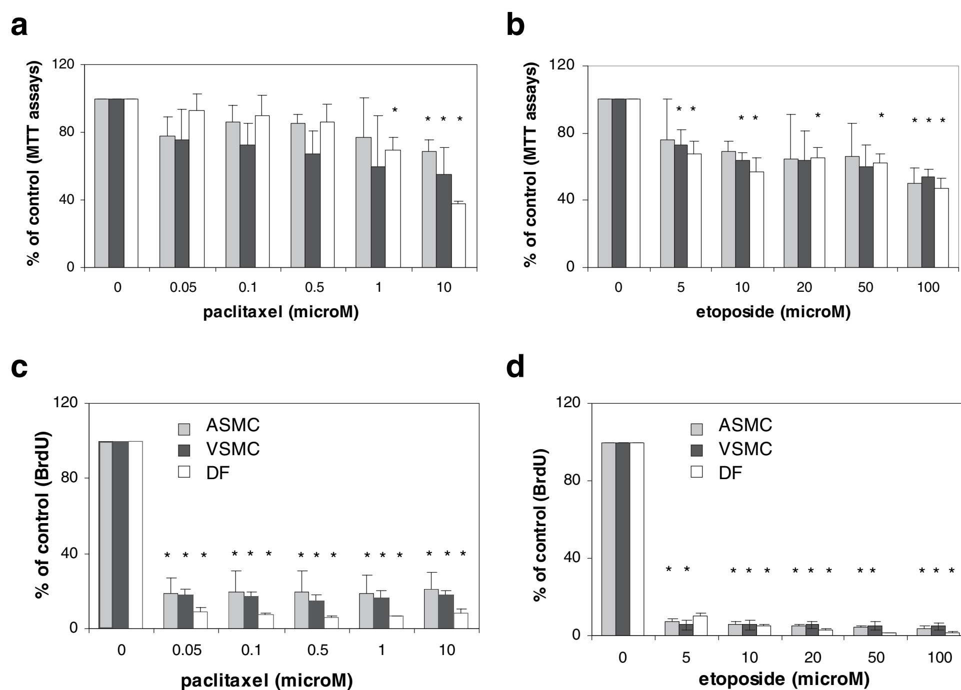

Fig. 1. Induction of apoptosis and suppression of cell proliferation in ASMCs, VSMCs, and DFs. **a**: Apoptotic response to paclitaxel treatment. ASMCs (gray bars), VSMCs (black bars), or DFs (white bars) were incubated with 0 (control), 0.05, 0.1, 0.5, 1.0, or 10 μM paclitaxel for 48 h and apoptosis determined by the MTT assay as described in the Materials and Methods. **b**: Apoptotic response to etoposide treatment. ASMCs (gray bars), VSMCs (black bars), or DFs (white bars) were incubated with 0, 5, 10, 20, 50, or 100 μM etoposide for 48 h and apoptosis was determined by the MTT assay. **c**: Suppression of cell proliferation by paclitaxel. Cells were treated as in panel (a) and labeled with BrdU to quantify cell proliferation as described in Materials and Methods. **d**: Suppression of cell proliferation by etoposide. Cells were treated as in panel (b) and labeled with BrdU to quantify cell proliferation. Statistical significance compared with the control is shown by * in all figures. Shown are the averages and standard deviations of four measurements.

Differences in the Role of MAPK Pathways Between ASMCs and VSMCs

Although the proliferation of ASMCs, VSMCs, and DFs were almost completely suppressed by paclitaxel or etoposide, disruption of mitochondrial potential only occurred to a limited extent (Fig. 1). These findings suggest that these cells are able to make compensatory adjustments that might lead to drug resistance after prolonged drug treatment. To understand the cellular signaling events responsible for the protection from apoptosis, we investigated several kinase pathways that have been reported to play important roles in cell proliferation and survival. We first investigated the MAPK pathway in ASMCs, VSMCs, and DFs in dose-response and time course analyses. For the time course studies, we used 100 nM paclitaxel or 10 μM etoposide, concentrations which are sufficient to induce proliferation arrest (Fig. 1c,d). For the dose-response studies, we chose 24 h for paclitaxel and 48 h for etoposide since the effect of etoposide appeared to be slower to develop than paclitaxel (data not shown). Cell lysates were analyzed by Western blotting using antibodies against p42/44 MAPK (to assess any changes in total protein levels) and phospho-specific p42/44 MAPK. The results indicated that these three cell types behaved very differently in their responses to the two apoptotic stimuli (Fig. 2). First, while the total amount of p42/44 MAPK did not change substantially in any cell type in the time-course study, phosphorylation of MAPK increased dramatically after incubation of ASMCs with either drug for 4 and 8 h. There was only a single band of phospho-MAPK at these two time points, which turns out to be p42 rather than p44 MAPK when aligned with the total MAPK blot (Fig. 2a). By 24 h, p42 MAPK phosphorylation had returned to unstimulated levels (Fig. 2a). In VSMCs, there was strong constitutive phosphorylation of p42/44 MAPK, which did not change

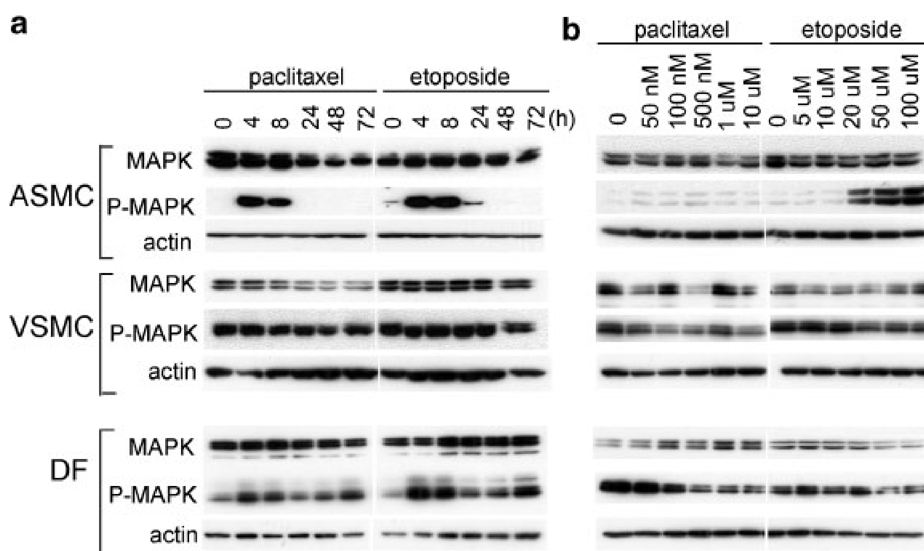

Fig. 2. The MAPK pathway in ASMCs, VSMCs and DFs. **a**: Time course analysis. ASMCs, VSMCs, and DFs were grown in their specific media to 80% confluence and then treated with 100 nM paclitaxel or 10 μM etoposide. Cells were harvested at the indicated time points for Western blot analysis using primary antibodies against p42/44 MAPK and phospho-(p42/44)MAPK. Western blots were developed using chemiluminescence. **b**: Dose-response analysis. ASMCs, VSMCs, and DFs at 80% confluence were treated with various concentrations of paclitaxel for 24 h or etoposide for 48 h and harvested for Western blotting as in panel (a). Each lane contained 30 μg of whole cell lysates. Immunoblotting with an antibody against smooth muscle β-actin was used as a control. Shown are representative Western blots from one of two independent experiments.

significantly after exposure to either drug. In DFs, there was a modest increase in phosphorylation of p42/44 MAPK at 4 and 8 h after treatment with either drug, which tended to diminish at subsequent time points. In the dose-response study, ASMCs showed no increase of p42/44 MAPK phosphorylation when treated with paclitaxel, but p42/44 MAPK phosphorylation dramatically increased with high concentrations of etoposide (Fig. 2b). In contrast, treatment of VSMCs and DFs with high concentrations of paclitaxel or etoposide resulted in modest decreases, rather than increases, in p42/44 MAPK phosphorylation (Fig. 2b).

Next we studied p38 MAPK, a member of the MAPK family closely involved in the stress response that has a pro-apoptotic effect upon activation [Xia et al., 1995]. The level of total p38 MAPK appeared to increase modestly in all cell types at 8–24 h after 100 nM paclitaxel treatment, and more substantially at 4–48 h after 10 μM etoposide treatment of ASMCs and DFs. No obvious phosphorylation of p38 MAPK was detectable in ASMCs after paclitaxel treatment and only transient phosphorylation was observed at 4 h after etoposide treatment (Fig. 3a). In VSMCs, there was modest constitutive phosphorylation of p38 MAPK that increased between 8 and 48 h after paclitaxel treatment, and disappeared by 72 h. With etoposide p38 MAPK phosphorylation in VSMCs was constant for the first 8 h, then began to steadily decrease. In DFs, there was a consistent increase in p38 MAPK phosphorylation after treatment with paclitaxel or etoposide, in contrast to ASMCs and VSMCs. The dose-response of ASMCs showed a small increase of p38 MAPK phosphorylation with paclitaxel, which was more prominent with etoposide (Fig. 3b). In contrast, VSMCs and DFs failed to show a dose dependence in p38 MAPK phosphorylation, except that very strong p38 phosphorylation was seen in DFs treated with 100 μM etoposide (Fig. 3b). Collectively, these studies show that ASMCs, VSMCs, and DFs displayed substantially different p38 MAPK responses to these two apoptotic stimuli, suggesting that activation of p38 MAPK may only be important in the response of ASMCs and DFs to paclitaxel and etoposide, but not in the response of VSMCs. Based on the pro-apoptotic effect of p38 MAPK in most cells [Xia et al., 1995; Chen et al., 2001], phosphorylation and activation of p38 MAPK should favor apoptosis. The absence of p38 MAPK phosphorylation in VSMCs in response to paclitaxel and etoposide suggests that VSMCs

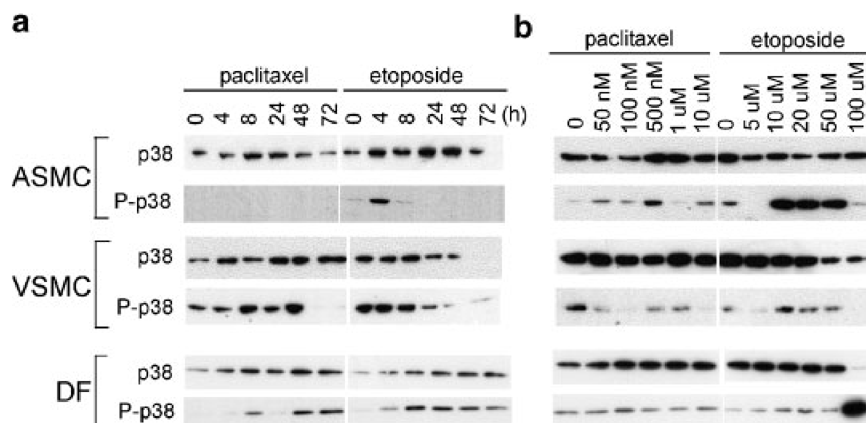

Fig. 3. Signaling events of p38 MAPK in ASMCs, VSMCs, and DFs. **a**: Time course analysis. ASMCs, VSMCs, and DFs at 80% confluence were treated with 100 nM paclitaxel or 10 μM etoposide and harvested at the indicated time points for Western blot analysis using primary antibodies against p38 MAPK and phospho-p38 MAPK antibodies. **b**: Dose-response analysis. ASMCs, VSMCs, and DFs at 80% confluence were treated with various concentrations of paclitaxel for 24 h or etoposide for 48 h and harvested for Western blotting as in (a). Each lane was loaded with 30 μg of whole cell lysates. Shown are representative Western blots from one of two independent experiments.

may differ from ASMCs in their pro-apoptotic signaling pathways.

To further explore the significance of the MAPK pathways, we tested the combination of paclitaxel with a MEK inhibitor, PD98059, in ASMCs and VSMCs. MTT assays were used to determine the degree of apoptosis 24 h after treatment. PD98059 alone had little effect on apoptosis and also did not significantly change the effect of paclitaxel on apoptosis in either ASMCs or VSMCs (Fig. 4a). Treatment of ASMCs with the p38 MAPK inhibitor PD169316 alone had no effect on apoptosis, but partially reversed the etoposide-induced apoptosis in ASMCs (Fig. 4b). These data are consistent with the effect of etoposide on increased p38 MAPK phosphorylation in ASMC (Fig. 3), and support the idea that p38 MAPK phosphorylation and activation are important for etoposide-induced apoptosis in ASMCs. The findings with VSMCs were essentially opposite to those seen with ASMCs. Treatment of VSMCs with PD169316 alone

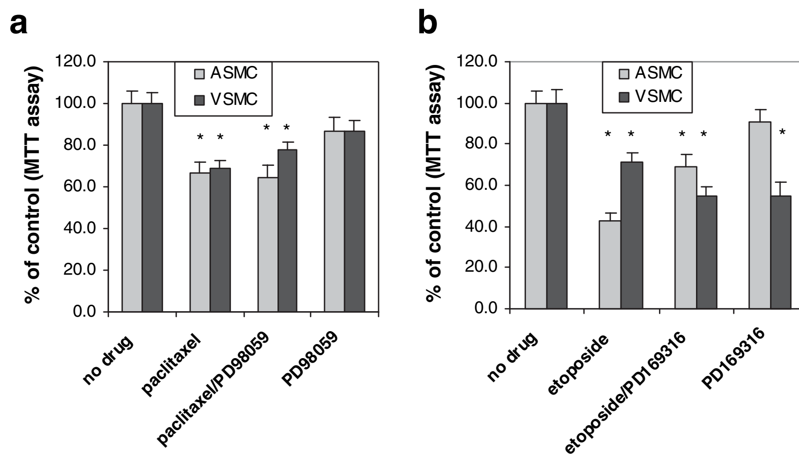

Fig. 4. Effect of MEK-specific inhibitor (PD98059) and p38 MAPK-specific inhibitor (PD169316) on ASMCs and VSMCs. ASMCs or VSMCs were treated with (**a**) 100 nM paclitaxel with or without MEK inhibitor PD98059 (6 μM) or (**b**) etoposide (10 μM) with or without p38 inhibitor PD169316 (10 μM) for 24 h. Cells were harvested for apoptosis analysis using the MTT assay. Shown are the means and standard deviations of ten measurements.

induced apoptosis in VSMCs to a greater extent than 10 μM etoposide alone (Fig. 4b). The addition of etoposide to PD169319 did not further increase apoptosis compared with PD169316 alone (Fig. 4b). The observation that blocking p38 MAPK activity by PD169316 induced apoptosis suggests that p38 MAPK may be a pro-survival factor in VSMCs, in contrast to its pro-apoptotic role in ASMCs.

Differences in the Role of the Akt Pathway Between ASMC and VSMC

Activation of PI3K in cells leads to generation of PIP3 that serves to activate Akt [Datta et al., 1999]. Akt is phosphorylated at Thr-308 and Ser-473 [Alessi et al., 1996, 1997]. Phosphorylation at Thr-308 is required for constitutive Akt activity, whereas phosphorylation at Ser-473 increases the activity of Akt and is commonly used as a marker to represent its activation by PIP3 [Chan and Tsichlis, 2001]. The results shown in Figure 5 show the effects of drug treatment on total Akt levels and levels of phospho-(Ser-473)Akt. In ASMCs treated with paclitaxel, the total amount of Akt increased at 4–24 h after treatment, but there was no increase in phospho-Akt at these same time points; after 24 h, phospho-Akt even decreased relative to unstimulated levels (Fig. 5a). These data indicate that paclitaxel treatment caused no activation of Akt in ASMCs. Dose-response experiments with paclitaxel also failed to show any increase in Akt activation (Fig. 5b). In contrast, when ASMCs were treated with etoposide, the total amount of Akt decreased but phospho-Akt increased until at least 72 h after treatment (Fig. 5a). Higher doses of etoposide (48 h) had no effect on total Akt levels, but reduced the levels of phospho-Akt (Fig. 5b). In VSMCs, treatment with either paclitaxel or etoposide caused minimal changes in phospho-Akt levels (Fig. 5a,b), suggesting that Akt is not involved in the apoptotic response of VSMCs to these agents. Interestingly, in DFs phosphorylation of Akt increased with time after paclitaxel treatment, but only increased transiently at 4–24 h after etoposide treatment (Fig. 5a). The dose-response studies of DFs to these drugs showed that higher concentrations of paclitaxel suppressed Akt phosphorylation, but increasing the etoposide dose to 20–50 μM significantly enhanced Akt phosphorylation (Fig. 5b). These results suggest that Akt may be activated in a compensatory mechanism that would suppress etoposide-induced apoptosis in ASMCs and both paclitaxel- and etoposide-induced apoptosis in DFs. Akt, however, appears to play no such role in VSMCs.

DISCUSSION

In this report, we describe the effects of paclitaxel and etoposide on proliferation and apoptosis in primary ASMCs, VSMCs, and DFs. We have focused on the Raf-1/MEK/(p42/44) MAPK, p38 MAPK and Akt pathways with the goal of understanding the compensatory signaling mechanisms for controlling proliferation and apoptosis in these cells. Both paclitaxel and etoposide were potent in suppressing cell proliferation as measured by the BrdU incorporation assay, but induced only modest apoptosis,

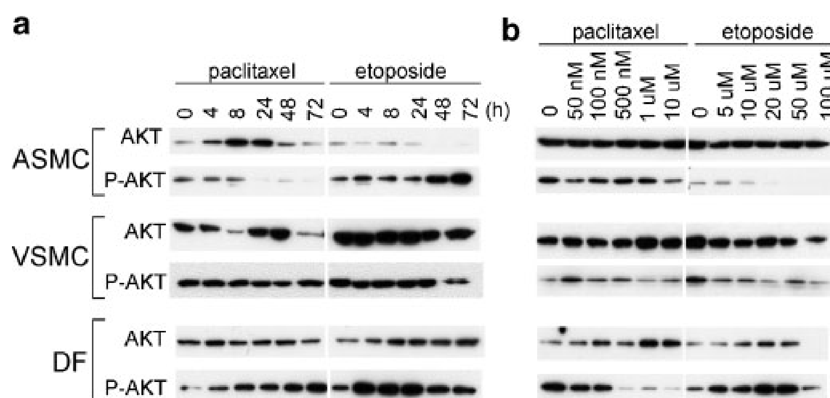

Fig. 5. Signaling events of Akt in ASMCs, VSMCs and DFs. **a**: Time course analysis. ASMCs, VSMCs, and DFs at 80% confluence were treated with 100 nM paclitaxel or 10 μM etoposide and harvested at the indicated time points for Western blot analysis using primary antibodies against Akt and phospho-(Ser473)Akt antibodies. **b**: Dose-response analysis. ASMCs, VSMCs, and DFs at 80% confluence were treated with various concentrations of paclitaxel for 24 h or etoposide for 48 h and harvested for Western blotting as in panel (a). Shown are

suggesting that compensatory mechanisms are invoked to prevent apoptosis in these cells. We analyzed the activation of specific pathways using antibodies that are specific for the phosphorylated forms of key downstream protein kinases. The transient increase in p42/44 MAPK phosphorylation after paclitaxel and etoposide treatment suggested the presence of cross-talk among signal transduction pathways that lead to p42/44 MAPK activation when chromosomes are damaged by either paclitaxel or etoposide. ASMCs may utilize this compensatory mechanism to prevent apoptosis and allow DNA repair to proceed. Since p42/44 MAPK activation is a pro-survival signal and could be a potential mechanism that mediates the resistance to anti-proliferative agents, a compound blocking the Raf-1/MEK/(p42/44) MAPK pathway might have a synergistic effect with either paclitaxel or etoposide in inducing apoptosis in ASMCs. Such an inhibitor combination might be ineffective for VSMCs, however, because minimal changes in p42/44 MAPK phosphorylation were detected in this cell type (Fig. 2). The compound PD98059 inhibits MEK [Alessi et al., 1995] and was used to test this hypothesis in ASMCs and VSMCs. If MEK (and downstream p42/44 MAPK) activation is a compensatory mechanism in preventing apoptosis induced by paclitaxel, we predicted that blocking this pathway by PD98059 might enhance the apoptotic effect of paclitaxel and etoposide in ASMCs, but not VSMCs and DFs. Using the MTT assay to assess apoptosis, the addition of PD98059, did not enhance the apoptotic effect of paclitaxel in ASMCs or in VSMCs (Fig. 4). The reason for the failure of PD98059 to enhance the apoptotic effects of paclitaxel and etoposide may be due to the transient effects of these agents on p42/44 MAPK phosphorylation, which was only observed at 4–8 h after treatment and was not sustained after this period (Fig. 2). These data thus argue against the rationale of using a combination of MEK inhibitors along with anti-proliferative agents to further inhibit the proliferation of SMCs.

Another protein kinase that may play a role in paclitaxel- and etoposide-induced apoptosis is p38 MAPK. p38 MAPK activation is often associated with apoptosis in response to stress, inflammation, and UV damage [Xia et al., 1995; Chen et al., 2001]. The activation of p38 MAPK upon treatment of ASMCs with high doses of etoposide was particularly prominent (Fig. 3b). Hence, blocking p38 with PD169316, a p38-specific inhibitor, could potentially suppress the apoptosis in ASMCs induced by anti-proliferative agents [English and Cobb, 2002]. This notion was indeed confirmed for ASMCs treated with etoposide, but not for VSMCs (Fig. 4b). In VSMCs, PD169316 actually induced apoptosis by itself. Therefore, the functional role of p38 in responding to anti-proliferative agents is dramatically different between AMSCs and VSMCs. These differences between ASMC and VSMC might be exploited in designing specific strategies to prevent vascular stenosis at specific sites, for instance in hemodialysis graft stenosis where stenosis is typically more prominent at the venous anastomotic site [Kanterman et al., 1995].

Finally, the activation of Akt following treatment with paclitaxel and etoposide appeared to be significant in ASMCs, but not in VSMCs. The activation of Akt is involved not only in regulating proliferation, but also cell death [Datta et al., 1999], and in the pathogenesis of diabetes [Zdychova and Komers, 2005]. Defective Akt2 signaling has been suggested to be a mechanism underlying the insulin resistance seen in Akt-knockout mice [Cho et al., 2001]. Alterations of Akt activity have been found in various tissues in diabetes and implicated in diabetic microvascular complications [Zdychova and Komers, 2005]. Akt signaling defects have been shown in endothelial cells of diabetic animal models, which may contribute to the development of endothelial dysfunction. Akt activity is increased in vascular SMCs, presumably due to higher plasma levels of insulin, leading to hyperplasia, a prominent histologic feature of dialysis vascular graft stenosis. Using immunohistochemical techniques, Chang et al. [2004] found a markedly greater proliferation index in the grafts of diabetic patients than those of non-diabetic patients, and suggested that anti-proliferative agents should be employed after angioplasty of the stenotic lesion, especially in diabetic patients. We observed that Akt became more activated in ASMCs after etoposide treatment, but Akt activation decreased after paclitaxel treatment. This finding, along with the known growth-promoting effects of Akt, suggests that paclitaxel may be a better anti-proliferative agent to use in preventing vascular stenosis than etoposide. In summary, the present study on the signaling events of SMCs treated with paclitaxel and etoposide provides a better understanding of the differences in

compensatory mechanisms in ASMCs, VSMCs, and DFs in response to cytotoxic agents, which should facilitate the development of better approaches to control SMC proliferation and induction of apoptosis to prevent stenosis in vascular grafts and coronary arteries.

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