Overexpression of p73 causes apoptosis in vascular smooth muscle cells

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Davis, Benjamin B., Yao Dong, and Robert H. Weiss. Overexpression of p73 causes apoptosis in vascular smooth muscle cells. Am J Physiol Cell Physiol 284: C16–C23, 2003; 10.1152/ajpcell.00211.2002.—Abnormal vascular smooth muscle (VSM) cell proliferation contributes to the development of atherosclerosis and its associated disorders, including angioplasty restenosis. The tumor-suppressor protein p53 has been linked to the development of atherosclerotic lesions, and its homolog, p73, is proving to have contrasting functions in a variety of tissues. As an outgrowth of our previous finding that p73 is increased in serum-stimulated VSM cells and human atherosclerotic tissue, we examined p73 overexpression in VSM cells to elucidate causality of p73 expression with growth response. Overexpression of p73 results in decreased cell cycle transit and is accompanied by apoptosis. The apoptotic changes in p73 overexpressing VSM cells are independent of p53 and are associated with a decrease in levels of p21waf1/cip1. In conjunction with our previous data finding that p73 is increased in serum-stimulated VSM cells, this work suggests a role for p73 in vascular proliferative diseases.

p21; p53; atherosclerosis; tet-off; proliferation

Since its cloning in 1979 (14, 16), the p53 gene and its associated tumor-suppressor protein have been extensively studied. The critical importance of p53 over evolutionary time is evident by its presence in more than 23 species ranging from mollusks to humans (26). But whereas p53 has been known for many years to function as an arrestor of cell cycle transit or a promoter of apoptosis, its newly described homologs, p73 and p63 (12), have been shown in some cases to have effects quite opposite to p53.

p73 has been studied extensively in cancer cells, where it has been shown under different conditions and with cell lines to be either pro- or anti-apoptotic (9, 11, 17, 21, 39), but the function of this protein in “normal” cell lines has not been adequately investigated. Given its homology to the tumor-suppressor protein 53, the finding that p73 is frequently overexpressed in human tumors (4, 29) suggests that this protein plays a growth regulatory role at least in this tissue, yet whether it contributes positively to cell growth or suppresses proliferation is not known. The issue is further complicated by the finding that the p73 protein, represented by its dual promoter and alternatively spliced forms, seems to have different, sometimes opposite, functions in different cell types. Thus it has been difficult to reconcile the similarities of these tumor proteins on downstream proteins with the findings, in particular, that p53 knockout mice develop spontaneous tumors at high frequencies in multiple tumors (10), whereas p73 knockouts show no increased susceptibility to spontaneous tumorigenesis (37).

p53 has been shown to be present in the vasculature (1) and exerts a growth-suppressive effect in human aortic vascular smooth muscle (VSM) cells (19). p53 is also markedly increased after balloon angioplasty of the rabbit iliac artery in a manner that parallels apoptosis (25). Thus this protein has been described as a possible “functional link” between VSM cell growth and apoptosis (19), as well as an important protein in the development of coronary restenosis (27). With the exception of our earlier work, in which we demonstrated that p73 is increased in serum-stimulated VSM cells (32), p73 has not been described otherwise in the vasculature and has been shown to be expressed only at very low levels in 51 other normal tissues (13). Although our previous work suggests that p73 is involved in some manner in vascular growth control, a
causal relationship between p73 expression and VSM cell growth has not been established. The nature of such a relationship is not obvious, due to the observed cell type differences in the function of p73, but it is likely that p73 is either causing VSM cell growth stimulation or is a homeostatic response in an attempt to attenuate this response.

Using a conditional overexpression model, we now show that p73 triggers a p73 expression-dependent inhibition of VSM cell S-phase entry (as a measure of cell cycle transit) and induction of apoptosis, independently of p53 but associated with a decrease in the level of the cyclin-dependent kinase inhibitor (and newly characterized “survival” protein) p21\(^{wt/cip}\). The fact that p73 is both induced in serum-stimulated VSM cells and is apoptosis promoting suggests that this response is a means by which vascular (and perhaps other) cells attenuate overexuberant VSM cell growth.

**MATERIALS AND METHODS**

**Materials.** Tet-off expression plasmids pBI-p73wt/EGFP (expressing wild-type p73) and tTA-ires-neo were obtained as kind gifts from Dr. Bert Vogelstein (Baltimore, MD). pTK-hygro was obtained from Clontech (Palo Alto, CA). Mouse monoclonal p21 antibody and human recombinant PDGF-BB were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal p53 and rabbit polyclonal caspase-3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). p73a antibody (Ab-1) was obtained from Oncogene Research Products (Boston, MA). Lipofectamine was obtained from Gibco (Grand Island, NY), and Fugene 6 was from Roche (Indianapolis, IN). Reagents for the enhanced chemiluminescence system and \[^{3}H\]thymidine were obtained from Amersham (Arlington Heights, IL). All other reagents were from Sigma (St. Louis, MO).

**Cell culture and proliferation assay.** Thoracic aortic smooth muscle cells were obtained from American Type Culture Collection (Rockville, MD), maintained as described (34), and human aortic smooth muscle cells were obtained from Clonetics (San Diego, CA). The established tet-off clones were maintained with the addition of 0.25 \(\mu\)g/ml doxycycline, 350 \(\mu\)g/ml G418, and 250 \(\mu\)g/ml hygromycin and were used in experiments between subpassages 15 and 25. After the indicated times of doxycycline withdrawal, \[^{3}H\]thymidine incorporation was assessed as previously described (34).

**Stable transfection of tet-off constructs.** The transfection protocol followed a method described by Yu et al. (38). A10 smooth muscle cells were first transfected with tTA-IRES-Neo using lipofectamine and selected by the addition of G418 (350 \(\mu\)g/ml). The G418-resistant cells were cotransfected with linealized pBI-p73wt/EGFP and pTK-hygro plasmid DNA at a molar ratio of 5:1 using Fugene 6 and further selected with the addition of hygromycin B at a concentration of 250 \(\mu\)g/ml. Single clones were selected by GFP expression. These hygromycin B-resistant cells were further selected by withdrawal or by a series reduction of doxycycline concentration in culture medium from 125 to 62.5 ng/ml, as evidenced by GFP expression under fluorescent microscope. The doxycycline-responsive cells were evaluated for p73 overexpression by Western blot analysis and for cell proliferation by \[^{3}H\]thymidine incorporation.

**Western blots.** Cells were grown to 80–90% confluence in six-well culture dishes. After withdrawal of doxycycline for the indicated times, cells were lysed, protein concentrations were determined by the Lowry method (Bio-Rad DC protein assay), and equal protein quantities were electrophoresed and Western blotted as previously described (34).

**Balloon angioplasty.** Lewis rats were anesthetized and the femoral artery was exposed. A balloon catheter was advanced by using a twisting motion until the balloon was under the inguinal ligament. Approximately 0.02 cc of saline was injected into the catheter to distend the balloon. The catheter was drawn back ~1 to 1.5 cm with constant pressure applied to the vessel. The pressure was then removed from the syringe and the catheter was reinserted into the artery, with a one-third turn (120°) of the catheter. The process was repeated for a total of three passes. At the end of the third ballooning, the catheter was removed so that only the tip of the catheter was occluding the vessel. After the indicated times, the rat was killed and the femoral artery was removed, lysed, and subjected to immunoblotting.

**Evaluation of nuclear morphology.** Cells were seeded in six-well culture dishes and treated as described. After removal of doxycycline for the times indicated (or no removal for controls), medium was aspirated and the cell culture dish was inverted over methanol for 10 min. The cells were then immersed in methanol for at least 10 min. Cells were stained in 1 \(\mu\)g/ml Hoechst 33258 in water with 1–2 g of nonfat dry milk. After being stained for 8–10 min, the cells were rinsed in water and completely dried. Nuclear morphology was visually evaluated by fluorescence microscopy.

**RESULTS**

p73 is increased in arteries subjected to balloon angioplasty. We have previously shown that p73, which is expressed at low levels in serum-starved VSM cells consistent with reports in other normal tissues (20), is increased after serum stimulation of VSM cells in vitro, as well as in human atherosclerotic plaque tissue in vivo (32). These data suggest that p73 is serving either a promitogenic or growth-suppressive role in this tissue. To further characterize the in vivo function of p73 in the rat tissue used in subsequent experiments, we examined expression of this protein in VSM cells from rat femoral arteries that had been damaged by balloon angioplasty. Two weeks after Lewis rats were subjected to balloon angioplasty, both the arteries subjected to angioplasty, as well as the contralateral control femoral arteries, were harvested and the homogenized tissue was electrophoresed and immunoblotted with p73a antibody. p73 in the arteries subjected to angioplasty was markedly increased compared with the level of this protein in the contralateral control arteries (Fig. 1), consistent with our previous findings of increased p73 levels in human atherosclerotic plaque tissue (32). This finding led us to hypothesize that p73 is functioning in some manner in growth or apoptotic control of the vasculature.

Because, in the case of the “injured” arteries and their controls described above, the entire vessel was harvested for immunoblotting, it is not known whether p73 was increased in the VSM cell layer or in other components of the artery. However, in light of our recent data indicating that p73 is increased in VSM cells of human atherosclerotic plaque (Yabes AP and Weiss RH, unpublished observations) and because it is the VSM cells that are mainly responsible for the
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\textbf{p}73 \textit{CAUSES APOPTOSIS IN VASCULAR SMOOTH MUSCLE CELLS}

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{p73 is increased after balloon angioplasty in rats. Anesthetized Lewis rats were subjected to angioplasty of the femoral artery. Two weeks later, the rats were killed and both the “injured” and contralateral control arteries were harvested, normalized for protein content, electrophoresed, and immunoblotted with p73 antibody. Two different samples of the ballooned arteries of 2 representative animals are shown. This experiment was repeated in 3 animals with identical results.}
\end{figure}

proliferative response in atherosclerosis (22), we focused on these component cells in subsequent studies.

\textit{Overexpression of p73 is independent of p53 but causes late attenuation of p21 levels.} Although we have shown that p73 is increased in human plaque tissue and in damaged rat arteries in vivo and in rat VSM cells stimulated with serum in vitro, it is not clear whether induction of this protein has a causal effect on proliferation of these cells, although it is tempting to infer from these data that p73 is functioning either to increase cell cycle transit or to limit cell proliferation. The former possibility is supported by the finding that p73 is increased in high-grade malignancies (6, 29) and the latter by reports that p73 causes apoptosis in certain cancer cells (28). To determine causality of p73 toward cell cycle events in a normal VSM cell line, we utilized a tetracycline-inducible expression system to conditionally overexpress full-length p73. After a tet-responsive VSM cell line had been successfully created (see MATERIALS AND METHODS), these cells were stably cotransfected with the wild-type p73 construct pBI-p73wt/EGFP obtained from Dr. Vogelstein (38) and pTK-hygro, and doxycycline withdrawal conditions were optimized in several clones to show maximal induction of p73 protein levels compared with noninduced cells. After 1 day of doxycycline withdrawal, mild fluorescence was observed, and by 3 days of doxycycline withdrawal, strong fluorescence appeared and persisted for at least 5 days. One clone was selected and used for subsequent experiments. In initial experiments, doxycycline was withdrawn for 5 days and expression of p73 was examined as a function of doxycycline dose (Fig. 2). At concentrations of doxycycline at 0.25 μg/ml or greater (including concentrations down to 0.0625 μg/ml, not shown), p73 levels were suppressed as assessed by immunoblotting, whereas, in the absence of doxycycline, p73 was present at high levels. p53, which has considerable sequence homology with p73, was not changed in cells overexpressing p73 compared with those not overexpressing p73 at all concentrations, including the absence of doxycycline (concentration = 0) when p73 is at high levels (Fig. 2). The tet-off-transfected cells were exquisitely sensitive to doxycycline, such that even very low concentrations of this antibiotic (down to 0.0625 μg/ml) inhibited expression of the transfected p73 gene as assessed by protein immunoblotting.

Although p73 and p53 activate similar target genes, endogenous p53 activity has been shown to be decreased in ovarian carcinoma cells hyperexpressing p73 (31), suggesting that p73 might act in an oncogenic manner in these cells that tolerate overexpression of this protein. In addition, p73 increases in lung cancer are independent of p53 gene alteration (29), suggesting that while p53 and p73 activate similar targets, they do so independently of each other. To determine the effect of p73 overexpression on p53 and a common downstream target, we examined levels of p53 and p21 as a function of time of p73 overexpression. Within 3 h after doxycycline withdrawal, p73 levels were increased, yet p53 levels were not significantly changed from 3 h until 5 days (Fig. 3), a result consistent with the dose response data (see Fig. 2). Surprisingly, after 5 days of doxycycline withdrawal, when p73 was maximally expressed, p21 levels were markedly attenuated. Thus, at least in VSM cells, p21 is not increased after p73 overexpression (in contrast to p53) and, furthermore, it is possible that p21 is functioning in its newly described antiapoptotic, prosurvival role in this setting, which is being reversed by p73 overexpression. Our data are thus consistent with the evolving concept that p21 is a negative regulator of apoptosis (reviewed in Ref. 8), as well as an Akt-mediated assembly factor described in our (33) and other (2, 24) laboratories. Furthermore, this result highlights yet another disparity between p53 and p73 signaling (15, 33).

\textit{Overexpression of p73 inhibits VSM cell cycle transit.} Because p73 overexpression has been shown to both correlate with high-grade malignancies and to induce apoptosis in certain cancer cell lines, events that are
difficult to reconcile with each other, we next determined the effect of p73 overexpression on the VSM cell cycle. Cell cycle transit of VSM tet-off cells grown continuously in serum-containing medium and overexpressing p73 after 5 days of doxycycline withdrawal (the time of maximal p73 overexpression) was markedly inhibited in an inverse doxycycline dose-dependent fashion, as measured by $[^3H]$thymidine incorporation (Fig. 4). The finding that $[^3H]$thymidine incorporation was partially inhibited despite minimal induction of p73 expression suggests that low levels of expression of p73 protein (i.e., not seen on the Western blot at the exposures used) are sufficient for cell cycle attenuation.

**p73 overexpression causes apoptosis.** Because inhibition of $[^3H]$thymidine incorporation by overexpressed p73 could result from a block in cell cycle transit and/or cell death, we next asked when maximal apoptosis occurs as a function of p73 overexpression. p73 induction of apoptosis has been previously described in cancer cells (reviewed in Ref. 28), in some cases through superactivation of E2F in the absence of p53 (9), but a death response to p73 overexpression in normal tissue or in vascular cells has not been described. Doxycycline was withdrawn from the tet-off cells to increase p73 expression for times ranging from 3 h to 5 days. The cells were stained with Hoechst 33258, and a representative field was examined under illumination conditions appropriate for either Hoechst or GFP fluorophores. Apoptosis was the most pronounced in those particular cells that have the maximal p73 overexpression, because the intensity of GFP fluorescence correlated closely with nuclear morphology of apoptosis (Fig. 5). These findings were not due to fluorescence of the Hoechst stain under GFP excitation wavelength because, in other experiments, cells that appeared to be apoptotic by visible light and were not stained with Hoechst also had the brightest GFP fluorescence (data not shown). In combination with plaque data (Fig. 1 and Ref. 32), our work suggests that p73 is increased in damaged vascular tissue and may function to limit an exuberant VSM cell-proliferative response often observed in such tissue.
DISCUSSION

Although the tumor-suppressor protein p53 has been extensively characterized due to the fact that it is mutated in the majority of human tumors, the function of its homolog p73 has been considerably more elusive. Various forms of p73 have been shown to be proapoptotic and growth suppressive (9, 11, 17, 39) or antiapoptotic (21), but this protein has been studied principally in malignant tissue, likely due to the findings of very low p73 expression in most normal tissues under basal conditions (13). Consistent with its putative, although elusive, role in cancer biology, p73 knockout mice were generated and had been expected to develop spontaneous malignancies similar to p53 knockout mice. However, p73 knockout mice do not show spontaneous tumors (37), and, despite the sequence similarity between p73 and p53, p73 mutations have been found, although infrequently, in human tumors (18). These data are difficult to reconcile with findings that p73 is overexpressed in certain cancers and that the level of its expression correlates with aggressiveness of these tumors. What the data do confirm is that the role of p73 in growth regulation, while likely profound, is not well understood.

Due to its original serendipitous discovery in chromosomal region that is frequently deleted in neuroblastoma (12), p73 studies have been largely confined to the field of cancer biology. Naturally, considerable excitement in the neurological cancer community was generated because of the idea, which turned out to be
an oversimplification, that p73 may be a p53 homolog, the deletion of which is pathogenic for neural cancers. A study of 51 different normal tissues showed very low p73 expression (13), but there is no information on growth-stimulated p73 expression in any normal tissue. The evolving role of p53 in vascular lesions, where its inhibition has been postulated to contribute to the development of coronary restenosis (27), led to our study of p73 in this tissue. Our finding that overexpression of p73 leads to apoptotic changes in VSM cells opens up a new area of investigation of this protein as a potential therapeutic signaling protein, in which this protein could ultimately prove useful in a clinical setting to limit aberrant proliferation of VSM cells.

p53 is increased after balloon angioplasty of rabbit arteries (1) in a manner that parallels apoptosis (25). The first (and thus far, only) published description of p73 in VSM cells was made in our laboratory, where the level of expression of p73 was shown to increase in serum-stimulated cells as well as in vivo in human atherosclerotic plaque tissue (32). Our finding in the present study that p73 overexpression causes apoptosis in VSM cells is an example of the similarity between the tumor-suppressor protein p53 and its homolog p73, yet the association of p73 with a decrease in p21 level at the time of maximal apoptosis is surprising and represents another significant divergence between p73 and p53 signaling. These data also suggest that p73 may utilize the survival or assembly factor function of p21 (2, 5, 15, 24, 33), a function that has not been demonstrated for p53. Interestingly, a similar, slight decrease in p21 was recently reported in cells in which p73β was overexpressed (3), but the authors of that study did not discuss this finding.

What is p73 doing in the vasculature? It has been well established that, after endothelial injury, it is the proliferation of VSM cells that contributes to the progression of the atherosclerotic state (22). Furthermore, the VSM cell proliferative response after coronary angioplasty is similar to the healing response of other tissues to injury, but in 25–50% of patients this response leads to coronary restenosis. A similar processes is believed to underlie hemodialysis vascular access stenosis. Based on our data in this study, we believe it is possible that p73 functions in vivo to attenuate the exuberant response of VSM cells to vascular injury. The proapoptotic action of p73 that we observed in VSM cells may parallel the action of p53 in cancer (1), yet the mechanism utilized by the two homologs in VSM cells may be disparate, involving p21 suppression in the case of p73. The findings that p53 and p73 knockout mice display quite disparate phenotypic abnormalities in terms of cancer susceptibility prove that different pathways are activated by these homologous proteins. Furthermore, p73 and p53 have been described as having competing effects (31, 36),
such that the downstream response of these transcription activators may be measured in their integrated effects on p21 or other cyclin-associated proteins.

The provocative possibility of the existence of a link between p73 and atherosclerosis may be seen in the finding that p73 maps to chromosome 1p36 (12), whereas a candidate human atherosclerosis susceptibility locus is present at 1p36–32 (35). A clinical connection between these two events could conceivably result from a loss of a normal apoptotic pathway associated with overexuberant VSM cell proliferation in response to atherosclerotic stimuli or tissue injury (as would occur during angioplasty), leading to the atherosclerotic condition. In addition, now that atherosclerosis, of course, begs further study.

Because VSM cell proliferation is the pathology underlining failure of angioplasty procedures, manipulation of p73 suggests possibilities for therapeutic intervention. Although p73 mutations have not been observed in human tumors, the possibility that decreased levels or mutation of p73 underlies atherosclerotic or other vascular proliferative diseases is provocative. Furthermore, supporting this gene as a potential gene therapeutic agent, p73 is assumed to replace or enhance the p53 protective role in p53-lacking cells (7), and there are now serious concerns regarding augmentation of p53 wild-type activity in mice (30). Thus p73 deserves further study as a tool to prevent VSM cell proliferation under conditions leading to atherosclerosis and other vascular diseases.

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