Autocrine Signaling through Ras Prevents Apoptosis in Vascular Smooth Muscle Cells in Vitro

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Vascular smooth muscle cell (SMC) apoptosis contributes to physiological and pathological vascular remodeling. Autocrine fibroblast growth factor (FGF) signaling promotes survival in SMC in vitro. Interruption of autocrine FGF signaling results in apoptosis that can be rescued by other growth factors such as PDGF (platelet-derived growth factor) or EGF (epidermal growth factor). Such heterologous growth factor rescue is prevented by pharmacological inhibition of MAPK, implicating signaling through Ras in mediating survival. This study was designed to test the hypothesis that signaling through Ras is both necessary and sufficient to mediate SMC survival in vitro. Recombinant adenoviruses encoding dominant-negative (RasN17) and constitutively active (RasL61) mutants of Ras were used. RasN17 blocks growth factor-mediated MAPK activation and can itself induce SMC apoptosis. RasN17 is synergistic with inhibition of autocrine FGF signaling in triggering apoptosis and prevents heterologous growth factor rescue. Conversely, RasL61 prevents apoptosis resulting from inhibition of autocrine FGF signaling. Rescue by RasL61 can be partially prevented by pharmacological inhibition of MEK or phosphatidylinositol 3-kinase, two downstream effectors of Ras. These results suggest that Ras signaling is both necessary and sufficient to mediate survival in SMC in vitro. Further work is required to determine how these signaling events are regulated in the context of vascular remodeling in vivo.

Apoptosis is a physiological process that is responsible for the regulated deletion of cells in many organs and tissues to establish and maintain proper organ architecture and physiology. Apoptosis is an essential feature of embryonic development and can be documented in a variety of tissues during postnatal life. In the vasculature, apoptosis in the smooth muscle compartment has been demonstrated in response to a sustained reduction in blood flow as a result of the switch from a fetal to a neonatal circulation, as well as in the settings of vascular injury and advanced atherosclerotic lesions. In the first context, the process of apoptosis is clearly a beneficial one and serves to maintain normal circulatory physiology in the face of major changes in hemodynamic requirements. In the latter two examples, it is less certain whether apoptosis represents an adaptive response or a pathological consequence.

We have previously demonstrated that autocrine FGF signaling serves to generate and sustain an anti-apoptotic survival signal in vascular smooth muscle cells in vitro (1, 2). Expression of a dominant-negative FGF receptor (DN-FGFR) interrupts an autocrine signaling loop in smooth muscle cells and triggers apoptosis. Under these circumstances, survival is critically dependent on reconstitution of anti-apoptotic signaling by stimulation of heterologous growth factor (EGF, PDGF) receptors. The ability of heterologous growth factors to rescue cells expressing DN-FGFR can be blocked by pharmacological inhibition of MEK (MAPK/extracellular signal-regulated kinase). These results suggest a pivotal role for growth factor signaling through Ras in mediating growth factor-dependent smooth muscle cell survival.

Ras is a complex multifunctional signaling molecule, receiving multiple inputs and provoking the activity of multiple downstream effectors (3). One of the best characterized Ras effector pathways is the activation of p42/p44 MAPK via a dual-specificity kinase cascade initiated by activation of the kinase Raf. Ras receives input from growth factor receptor tyrosine kinases via guanine nucleotide exchange factors (4, 5) and may receive activating input from heterotrimeric G protein-coupled receptors through similar exchange factors or, less directly, through activation of cytoplasmic tyrosine kinases of the Src family (6). Ras can also activate lipid kinases, especially phosphatidylinositol 3-kinase (7), as well as interact with other divergent effector pathways involving RalGDS (8), c-Jun N-terminal kinase via other dual specificity kinase cascades (9), and related small monomeric G proteins of the Rho family (10, 11).

Various reports in the literature have documented an important role for Ras and its effector pathways in influencing cellular behavior and, in particular, cell survival. Signals responsible for survival in the pheochromocytoma cell line PC12 may depend on activation of phosphatidylinositol 3-kinase, and dominant-negative inhibition by RasN17 does not appear to affect survival (12). Dominant-negative inhibition by RasN17 alone may even be sufficient to protect PC12 cells from apoptosis (13), but the data from these two studies conflict on this point. In transformed fibroblasts, radiation-induced apoptosis can be prevented by expression of constitutively active Ras (14, 15). Conversely, expression of constitutively active Ras is associated with enhanced sensitivity to tumor necrosis factor-α-induced apoptosis (16). Apoptosis induced by tumor necrosis

*This work was supported by grants from the National Institutes of Health (HL02859 to J. C. F.); the American Heart Association (965055N to J. C. F.) and its Southeastern Pennsylvania affiliate (to T. M.), and the University of Pennsylvania Research Foundation (to J. C. F.). The costs of publication of this article were defrayed in part by the payment of page charges. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: FGF, fibroblast growth factor; bFGF, basic FGF; DN-FGFR, dominant-negative FGF receptor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; m.o.i., multiplicity of infection; P38K, phosphatidylinositol 3-kinase; SMC, smooth muscle cells; TUNEL, terminal deoxynucleotidyl transferase nick-end labeling.
factor-α in transformed fibroblasts is prevented by bFGF, and this protection is abrogated by expression of Ras\textsuperscript{N17} (17). Many pathways inducing apoptosis are inhibited by Bcl-2, and overexpression of constitutively active Ras induces Bcl-2 and protects from apoptosis caused by growth factor withdrawal in hematopoietic cells (18).

The present study was designed to test whether Ras activity is both necessary and sufficient to mediate vascular smooth muscle cell survival in vitro and to probe candidate signaling pathways downstream of Ras that might be responsible for this activity. The regulation of smooth muscle apoptosis may be as important as migration and proliferation in a variety of disease processes. By determining the signaling mechanisms responsible for mediating smooth muscle cell survival, we may ultimately be able to manipulate these mechanisms in vivo for the therapeutic benefit of patients with vascular disease.

MATERIALS AND METHODS

Recombinant Adenoviruses—The construction and preparation of the recombinant adenoviruses used in these experiments have been described previously. These include constructs encoding the dominant-negative FGF receptor (Ad.DN-FGFR) (2), b-galactosidase (Ad.CMV\textit{lacZ}, gift of Dr. James Wilson, University of Pennsylvania) (19), and the Ras mutants Ras\textsuperscript{N17} and Ras\textsuperscript{L61} (gift of Dr. Joe Nevins, Duke University) (20). Working stocks of virus were prepared by large scale infection of 293 cells with purified seed stocks as described previously and stored in aliquots at -80 °C. Ad.DN-FGFR or its lac\textit{Z} control were used to infect cells at an m.o.i. of 250. The Ras adenoviruses were used at the m.o.i. indicated in the figures (50-500) or in the figure legends.

Cell Culture and Western Blotting—Rat aortic smooth muscle cells (gift of Dr. Gary Owens, University of Virginia) between passages 9 and 12 were cultured and infected with recombinant adenoviruses as described previously (1). Where indicated, cells were treated with the MEK inhibitor PD98059 (Alexis Corp., San Diego, CA) or the phosphatidylinositol 3-kinase inhibitors wortmannin or LY294002 (Alexis Corp.). Cell counts were obtained by hand counting of trypsinized cell suspensions using a hemacytometer. Tissue culture reagents were from Life Technologies, Inc. unless otherwise specified.

Analysis of p42/p44 MAPK, p38 MAPK, and c-\textit{akt} Activation (Phosphorylation)—To determine the phosphorylation state of p42 erk1 and p44 erk2 mitogen-activated protein kinases (MAPK), a Western blot assay was employed as described previously (2). Smooth muscle cells were serum deprived for 48 h. During this period of serum deprivation, cells were either sham-infected or infected with recombinant adenoviruses to allow transection expression for 48 h prior to ligand stimulation and analysis. Cells were exposed to 25 ng/ml recombinant bFGF for various times, harvested by direct lysis in SDS-polyacrylamide gel electrophoresis sample buffer containing protease and phosphatase inhibitors, assayed for total cell protein (Bio-Rad), and up to 8 µg of cell protein per sample was separated by SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA). Membranes were probed first with a polyclonal antibody specific for the phosphorylated forms of p42/p44 MAPK and then re-probed with a different polyclonal antibody recognizing both the phosphorylated and dephosphorylated forms of MAPK (New England Biolabs, Beverly, MA). Similar assays were performed using paired antibodies directed against p38 MAPK and c-\textit{akt} (New England Biolabs) under the same conditions.

TUNEL Staining—Quantitative terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assays were performed as described previously (2) using a fluorescein isothiocyanate-conjugated dUTP kit according to the manufacturer’s instructions (Roche Molecular Biochemicals).

RESULTS

We have previously demonstrated that interruption of an autocrine FGF signaling loop in vascular smooth muscle cells triggers apoptosis. In the case of expression of a DN-FGFR, cell death can be prevented by other peptide growth factors. The receptors for these growth factors activate some signaling molecules in common with FGFRI. The MEK inhibitor PD98059 can block rescue by these other growth factors, implicating the MAPK activation cascade in mediating a survival signal in vascular smooth muscle cells (2). Heterologous activation of the MAPK pathway may be sufficient to mediate survival in this context, but we considered whether other signaling pathways downstream of Ras might also be involved. Activation of phosphatidylinositol 3-kinase (PI3K) has also been identified as an important downstream effector of Ras, and PI3K (and the downstream kinase c-\textit{akt}) has been implicated in promoting cell survival in other systems. As an initial test of the hypothesis that activation of PI3K might contribute to survival in cultured vascular smooth muscle cells, cells expressing DN-FGFR were treated with the growth factors bFGF (25 µg/ml), EGF (100 µg/ml), or PDGF (25 µg/ml) in the absence or presence of wortmannin (100 nM), an inhibitor of PI3K. Fig. 1 shows that wortmannin effectively blocks the ability of heterologous growth factors (EGF, PDGF) to rescue cells expressing DN-FGFR to an extent comparable with 30 µM PD98059. This result further implicates signaling through Ras in activating effector signaling pathways promoting survival in vascular smooth muscle cells. To test directly whether Ras activity is both sufficient and necessary to mediate survival in vascular smooth muscle cells, we employed adenoviral expression of two mutants of Ras, one of which is constitutively active (Ras\textsuperscript{L61}) and one of which acts as a dominant-negative (Ras\textsuperscript{N17}). Expression of these Ras mutants was characterized biochemically in terms of the ability to activate or block, respectively, the downstream effectors MAPK and c-\textit{akt}. Expression of the constitutively active and dominant-negative Ras mutants was also characterized biologically in terms of their ability to promote or antagonize the survival of cells expressing DN-FGFR.

To demonstrate the biochemical consequences of Ras mutant expression, we used Western blot assays for phosphorylation of p42/p44 MAPK (erk1/erk2) and c-\textit{akt} as evidence for activation of the MAPK and PI3K pathways, respectively. Rat aortic smooth muscle cells were cultured under conditions designed to reveal whether Ras activity was either necessary or sufficient to lead to activation of these pathways and to promote or antagonize cell survival. To test whether expression of Ras\textsuperscript{L61} was sufficient to activate the MAPK and c-\textit{akt} pathways in SMC, cells were infected with adenoviruses encoding lac\textit{Z} (as control) or DN-FGFR, with or without concomitant infection with Ras\textsuperscript{L61}, and maintained in growth medium (10% fetal bovine serum). In addition, cells were treated with the MEK inhibitor PD98059 (30 µM), the PI3K inhibitor wortmannin (100 nM), or a combination of these inhibitors. After 48 h, cell lysates were analyzed for MAPK and c-\textit{akt} activation. Fig. 2 shows that Ras\textsuperscript{L61} expression results in constitutive MAPK and c-\textit{akt} phosphorylation, regardless of whether cells were also expressing DN-FGFR. The addition of the MEK inhibitor PD98059 substantially inhibited the ability of Ras\textsuperscript{L61} expression to result in constitutive MAPK phosphorylation. Compared with a clear signal of phosphorylated c-\textit{akt} in cells expressing Ras\textsuperscript{L61}, addition of wortmannin markedly reduced the degree of c-\textit{akt} phosphorylation. Treatment with PD98059 also reduced the level of c-\textit{akt} phosphorylation, and wortmannin treatment led to a reduced signal for phosphorylated MAPK. Combining the inhibitors resulted in levels of phosphorylation similar to treatment with either inhibitor alone; similar results were obtained when the PI3K inhibitor LY294002 (10 µM) was substituted for wortmannin (data not shown).

To characterize the biochemical consequences of blocking Ras activity, cells were infected with recombinant adenoviruses encoding either the dominant-negative mutant Ras\textsuperscript{N17} or lac\textit{Z} as a control and allowed to express the transgenes for 48 h under serum-free conditions. Following stimulation with bFGF, EGF, or PDGF the cells were analyzed for MAPK and c-\textit{akt} activation. These data demonstrate that Ras\textsuperscript{N17} blocks
Ras Prevents SMC Apoptosis

Fig. 1. Inhibitors of MEK and PI3K antagonize the ability of heterologous growth factors to rescue apoptosis triggered by dominant-negative FGFR expression. A, rat aortic SMC expressing a dominant-negative FGF receptor (DNFR, black bars) or control lacZ (LZ, shaded bars) were treated with vehicle alone (−GF), bFGF (25 ng/ml), PDGF (25 ng/ml), or EGF (100 ng/ml). Cells were further treated with either vehicle (−PD, hatched black bars) or MEK inhibitor PD98059 (+PD, hatched white bars; 30 μM). The pan-caspase inhibitor, anti-apoptotic agent zVAD-fmk (+zVAD, 100 μM) was included as an additional negative control. After 2 days in culture under these conditions, the proportion of apoptotic cells was measured by counting the percentage of 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei under the fluorescence microscope. Data points represent the mean ± S.D. of triplicate samples. This experiment was repeated three times with similar results. B, rat aortic SMC expressing a dominant-negative FGF receptor (DNFR) or lacZ (LZ, control) were treated with vehicle alone (−GF), bFGF, PDGF, or EGF. Cells were further treated with either vehicle (−W) or the PI3K inhibitor wortmannin (+W, 100 nM). The pan-caspase inhibitor, anti-apoptotic agent zVAD-fmk (+zVAD, 100 μM) was included as an additional negative control. After 2 days in culture under these conditions, the proportion of apoptotic cells was measured by counting the percentage of 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei as described for A. Data points represent the mean ± S.D. of triplicate samples. This experiment was repeated three times with similar results.

To test whether Ras activity was itself sufficient to mediate SMC survival in this model, cells expressing DN-FGFR (or lacZ as control) were co-infected with the virus encoding RasL61 in the absence or presence of biochemical inhibitors. Fig. 2 shows that RasL61 is able to prevent apoptosis triggered by DN-FGFR (−adds) as measured by a quantitative assessment of the proportion of apoptotic cells using TUNEL staining. Proliferation (as measured by cell count) is not increased in lacZ-expressing control cells that also express RasL61 (data not shown). This is consistent with the observation that Ras activity alone is insufficient to provoke proliferation (and the transformed phenotype) in nonimmortalized cells (20). As a further demonstration that Ras signaling to MAPK is involved in SMC survival, in cells expressing DN-FGFR (or lacZ as control) and RasL61, the MEK inhibitor PD98059 partially prevents RasL61 from rescuing cells expressing DN-FGFR. We considered that other signaling pathways activated by Ras such as PI3K and the downstream kinase c-akt might contribute to mediating a survival signal in this model. To test this possibility, cells expressing DN-FGFR (or lacZ) and RasL61 were also treated with the PI3K inhibitor wortmannin or a combination of PD98059 and wortmannin. Fig. 5 also shows that wortmannin partially blocks the ability of RasL61 to rescue cells from apoptosis triggered by DN-FGFR and that the effects of PD98059 and wortmannin may be additive. This last observation must be interpreted cautiously, however, in light of the results of the biochemical readouts using these inhibitors in this experimental system. Of additional interest in this experiment is the observation that control cells expressing lacZ with or without RasL61 display slightly increased apoptosis in the presence of PD98059 or wortmannin, suggesting that these signaling pathways are important to cell survival under basal conditions (i.e. in the absence of DN-FGFR or mutant Ras expression).

We next determined whether Ras activity was necessary for SMC survival in the absence of any other influence (i.e. with the autocrine FGF signaling loop intact but without additional growth factors present). Fig. 6 shows that RasN17 expression alone provokes SMC apoptosis in a dose-dependent manner. Using a dose of RasN17 adenovirus that inhibits MAPK or c-akt phosphorylation (Figs. 3 and 4) and triggers apoptosis (Fig. 6),
cells were co-infected with DN-FGFR (or lacZ) and RasN17 to determine whether RasN17 either amplified the pro-apoptotic influence of DN-FGFR expression or blocked the ability of PDGF or EGF to rescue cells expressing DN-FGFR. Fig. 7 shows that even low levels of RasN17 enhance the pro-apoptotic consequences of interrupting the autocrine FGF signaling loop with DN-FGFR and prevent the ability of PDGF or EGF to rescue DN-FGFR-triggered apoptosis.

**DISCUSSION**

Previously published experiments from our laboratory established the importance of an autocrine FGF signaling loop in promoting survival in cultured rat aortic smooth muscle cells (1, 2), and in vivo experiments in the rat carotid injury model are consistent with the physiological relevance of this signaling loop.

FIG. 3. Activation of p44/p42 MAPK by bFGF, PDGF, or EGF is antagonized by the dominant-negative mutant RasN17. Rat aortic smooth muscle cells were sham-infected (Un) or infected with recombinant adenoviruses expressing lacZ (LZ, m.o.i. 500) or RasN17 at an increasing m.o.i. (maximum 500) and incubated in serum-free medium for 48 h prior to treatment with vehicle (−) or bFGF (25 ng/ml), PDGF (25 ng/ml), or EGF (100 ng/ml) (+). Extracts were prepared and probed by Western blotting for the phosphorylation state of p44/p42 MAPK activated by bFGF (top two panels), PDGF (middle two panels), or EGF (bottom two panels). Each pair of panels was sequentially probed with an antibody recognizing only the phosphorylated forms of p44/p42 MAPK (anti-PMAPK) followed by an antibody recognizing the total MAPK signal (anti-TMAPK).

FIG. 4. Activation of c-akt by bFGF, PDGF, or EGF is antagonized by the dominant-negative mutant RasN17. Rat aortic smooth muscle cells were sham-infected (Un) or infected with recombinant adenoviruses expressing lacZ (LZ, shaded bars) alone or co-infected with a recombinant adenovirus expressing the constitutively active mutant RasL61 (DNRas, hatched bars; LZ/Ras, hatched white bars) were treated with vehicle alone (−adds) or the MEK inhibitor PD98059 (+PD, 30 μM), the PI3K inhibitor wortmannin (+Wort, 100 nM), or both (+PD/+Wort). After 2 days in culture under these conditions, the proportion of apoptotic cells was measured by quantitative TUNEL staining. Data points represent the mean ± S.D. of triplicate samples. This experiment was repeated three times with similar results.

FIG. 5. Constitutive activation of the Ras pathway by RasL61 blocks apoptosis triggered by expression of a dominant-negative FGF receptor, and rescue is antagonized by inhibitors of MEK (PD98059) and PI3K (wortmannin). Rat aortic SMC expressing a dominant-negative FGF receptor (DN, black bars) or lacZ (LZ, shaded bars) alone or co-infected with a recombinant adenovirus expressing the constitutively active mutant RasL61 (DNRas, hatched bars) were treated with vehicle alone (−adds) or the MEK inhibitor PD98059 (+PD, 30 μM), the PI3K inhibitor wortmannin (+Wort, 100 nM), or both (+PD/+Wort). After 2 days in culture under these conditions, the proportion of apoptotic cells was measured by quantitative TUNEL staining. Data points represent the mean ± S.D. of triplicate samples. This experiment was repeated three times with similar results.
to mediate survival under these circumstances. Data presented in this report directly address the hypothesis that Ras activity is both necessary and sufficient to mediate vascular smooth muscle cell survival in vitro. The dominant-negative mutant Ras$^{N17}$ was able to block both biochemical evidence of growth factor activation of the Ras pathway (MAPK phosphorylation) and PDGF or EGF rescue of DN-FGFR-triggered apoptosis. Indeed, Ras$^{N17}$ by itself was able to trigger apoptosis in the absence of other manipulations, and this consequence of Ras blockade was insensitive to added growth factors. Conversely, expression of a constitutively active Ras$^{L61}$ mutant led to biochemical evidence of constitutive activation of both MAPK and c-akt, downstream effectors of Ras, even in the presence of DN-FGFR co-expression. Expression of Ras$^{L61}$ was also able to promote SMC survival in the presence of DN-FGFR co-expression in the absence of an influence on cell proliferation. Pharmacological inhibitors of these downstream Ras effector pathways substantially abrogated the ability of Ras$^{L61}$ to promote survival under these conditions. These experiments provided unanticipated evidence for possible cross-talk between the MAPK and c-akt pathways in the biochemical read-outs (phospho-specific Western blots). PD98059, considered a selective MEK inhibitor, was able to reduce the degree of phosphorylation of c-akt in response to Ras$^{L61}$ expression. Similarly, wortmannin (or LY294002), a reportedly selective PI3K inhibitor, was able to reduce the signal of phospho-MAPK in response to Ras$^{L61}$. These data are consistent with the observations of other investigators examining the activity of these signaling pathways in the presence of these kinds of pharmacological inhibitors (23–28), although it should be noted that such cross-talk appears to be cell type- and context-specific. Supported by these observations of other investigators, the present results suggest that, in the context of strong, constitutive activity of Ras, persistent activation of either the MAPK or c-akt pathways may be co-dependent in vascular smooth muscle cells. These experiments were not designed to definitively characterize the pathways downstream of Ras as protecting smooth muscle cells from apoptosis. However, the results strongly suggest that a survival signal or signals, generated in a tonic manner by Ras, result from autocrine FGF signaling in vascular smooth muscle or, in its absence, from similar tyrosine kinase growth factor receptors signaling through Ras.

Whether Ras activity promotes or antagonizes survival appears to depend on the cell type and the signaling mechanism responsible for initiating apoptosis. For example, Ras promotes cell cycle progression, and therefore, in the case of apoptosis being triggered by circumstances such as DNA damage by ionizing radiation, which normally results in cell cycle arrest and attempts at DNA repair, constitutive activation of Ras promotes apoptosis. On the other hand, in models of growth factor deprivation characterized by the interruption of signaling through receptors that activate the Ras pathway, ectopic stimulation of this pathway (either through heterologous growth factor receptors or expression of a constitutively active Ras) supports cell survival. Unfortunately, elucidation of the actual mechanisms responsible for regulating cell survival through the varied effector pathways downstream of Ras may not be quite so straightforward, as contrary examples to these observations about the role of Ras vis-à-vis cell cycle progression versus trophic factor deprivation have also been reported. Part of the reason for these different results could be that Ras activates a variety of signaling pathways other than the MAPK cascade (29–31). These other pathways include PI3K and the related activation of the kinase c-akt, which itself has been implicated in mediating anti-apoptotic signals, as well as the c-jun N-terminal kinase pathway via MAPK kinase kinase 5.
Ras is also reported to activate related small monomeric G proteins such as Rac, Rho, cdc42, and RalGDS. The final outcome in a particular cell type under specific circumstances may well depend on a fine balance between signaling through various effector pathways receiving input from Ras and other signaling pathways subserved by alternative mechanisms, such as other protein kinase cascades, cyclic nucleotides, lipid kinases, phospholipases, phosphatases, or neutral lipids such as ceramide.

If the outcome of such a balance in signal transduction mechanisms in a particular cell type is regulated in a physiological manner, how can we relate the results presented here to the environment of the vascular smooth muscle cell in vivo? Apoptosis in the vascular smooth muscle compartment has been documented under a variety of circumstances, but these have as a common theme the context of vascular remodeling. Little is known about how alterations in growth factors and potentially pro-apoptotic cytokines might affect smooth muscle cell survival in the context of vascular remodeling in these different scenarios in vivo, but some important clues are provided by studies of smooth muscle cell apoptosis in vitro. The current study and previous work from our laboratory supply evidence that smooth muscle cells possess an autocrine mechanism to provide trophic support in a manner reminiscent of other mod-

Acknowledgments—The authors thank Dr. Joseph Nevins (Duke University) for kindly providing the recombinant adenoviruses encoding the RasN17 and RasL61 mutants, Dr. Gary Owens (University of Virginia) for rat aortic vascular smooth muscle cells, and Dr. Judith Meinkoth (University of Pennsylvania) for helpful discussions and critical review of the manuscript. Ann S. McNamara provided valued technical assistance.

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