Fas cross-linking induces apoptosis in human airway smooth muscle cells

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Hamann, Kimm J., Joaquim E. Vieira, Andrew J. Halayko, Delbert Dorschied, Steven R. White, Sean M. Forsythe, Blanca Camoretti-Mercado, Klaus F. Rabe, and Julian Solway. Fas cross-linking induces apoptosis in human airway smooth muscle cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L618–L624, 2000.—Hypertrophy and hyperplasia lead to excess accumulation of smooth muscle in the airways of human asthmatic subjects. However, little is known about mechanisms that might counterbalance these processes, thereby limiting the quantity of smooth muscle in airways. Ligation of Fas on the surface of vascular smooth muscle cells and nonmuscle airway cells can lead to apoptotic cell death. We therefore tested the hypotheses that 1) human airway smooth muscle (HASM) expresses Fas, 2) Fas cross-linking induces apoptosis in these cells, and 3) tumor necrosis factor (TNF)-α potentiates Fas-mediated airway myocyte killing. Immunohistochemistry using CH-11 anti-Fas monoclonal IgM antibody revealed Fas expression in normal human bronchial smooth muscle in vivo. Flow cytometry using DX2 anti-Fas monoclonal IgG antibody revealed that passage 4 cultured HASM cells express surface Fas. Surface Fas decreased partially during prolonged serum deprivation of cultured HASM cells and was upregulated by TNF-α stimulation. Fas cross-linking with CH-11 antibody induced apoptosis in cultured HASM cells, and this effect was reduced by long-term serum deprivation and synergistically potentiated by concomitant TNF-α exposure. TNF-α did not induce substantial apoptosis in the absence of Fas cross-linking. These data represent the first demonstration that Fas is expressed on HASM and suggest a mechanism by which Fas-mediated apoptosis could act to oppose excess smooth muscle accumulation during airway remodeling in asthma.

asthma; airway remodeling; cytokines

Recent studies (8, 16, 23) indicate that both hypertrophy and hyperplasia lead to excess accumulation of smooth muscle in the airways of asthmatic humans. However, little is known about mechanisms that might counterbalance these remodeling processes and limit the quantity of smooth muscle in airways. In other organs, tissue architecture reflects the balance of cell accumulation through growth or proliferation versus cell loss through shedding or cell death (4, 25).

Apoptosis is a form of programmed cell death that represents a pathway by which tissues can eliminate unhealthy, harmful, or excess cells. Fas, a member of the tumor necrosis factor (TNF)-α receptor family, plays a dominant role in the initiation of cell death in many cell types (1, 27). For example, Fas is expressed on vascular smooth muscle cells, and its ligation appears to induce myocyte apoptosis in atheromatous plaques (3, 5, 9, 10).

Our group (13, 14, 26) and others (31, 33, 35) have identified Fas-mediated apoptosis as a mechanism that may limit survival of T cells, eosinophils, and bronchial epithelial cells in airways. We postulated that Fas-mediated apoptosis might also regulate airway smooth muscle remodeling by acting to reduce airway smooth muscle cell number. In this study, we tested the hypotheses that 1) human airway smooth muscle (HASM) expresses Fas, 2) Fas cross-linking induces apoptosis in these cells, and 3) TNF-α potentiates Fas-mediated airway myocyte killing.

Methods

Immunohistochemical detection of Fas in HASM. Normal-appearing central airways were obtained from lungs surgically resected for lung carcinoma in four patients treated at the Krankenhaus Grosshansdorf (Grosshansdorf, Germany). Fas protein was detected with CH-11 primary anti-Fas IgM antibody (19, 21) and standard immunoperoxidase staining; serial sections were similarly processed with mouse IgG as a negative control. FITC-conjugated mouse anti-α-smooth muscle actin (Sigma) was used to confirm smooth muscle localization in additional serial sections; isotype-matched mouse IgG served as a negative control, and nuclei were counterstained with Hoechst 33348.

Cell culture. HASM cells were obtained by enzymatic digestion from smooth muscle bundles dissected from the lower tracheae and central bronchi of human lungs obtained either from surgical specimens or from organ donors whose lungs could not be used for lung transplant. Cells from four individuals were studied in each experiment described below, except as noted. Smooth muscle cells were dissociated from tissue by digestion with collagenase (600 U/ml), elastase type IV (1 U/ml), and protease type XXVII (2 U/ml) for 30–45 min at 37°C followed by gentle mechanical trituration and were cultured in DMEM supplemented with fetal bovine serum (20%), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), amphotericin B (0.25 µg/ml), and nonessen-
tial amino acids under 5% CO$_2$ at 37°C. Cells were passaged at a 1:3 dilution by digestion with 0.05% trypsin and 0.5 mM EDTA and were maintained thereafter in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and nonessential amino acids. Cells were studied at passage 4, at which time at least 85% of cells stained prominently for α-smooth muscle actin.

Flow cytometry. Confluent passage 4 HASM cells from each donor were maintained in serum-free DMEM supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), and selenium (5 ng/ml) for 0–8 days, and some were stimulated with TNF-α (10 ng/ml) during days 1–8 of serum deprivation. Cells were lifted in calcium- and magnesium-free phosphate buffer containing 0.5 mM EDTA, then stained with propidium iodide (PI; 50 µg/ml) and FITC-conjugated anti-Fas (IgG) monoclonal antibody (DX2; PharMingen, San Diego, CA) (6, 15) or mouse isotypic (IgG) control antibody. Flow cytometry was performed with a Becton Dickinson FACScan, and results were gated to exclude dead (PI-positive) cells.

Induction of apoptosis in cultured HASM cells. HASM cells from each donor were grown to confluence on uncoated glass coverslips and were then exposed to DMEM plus insulin, transferrin, and selenium for 24 h before induction of apoptosis. At this time, the following were added to the culture medium: 1) no additive (control condition), 2) control IgM antibody (1 µg/ml), 3) TNF-α (10 ng/ml), 4) CH-11 monoclonal anti-Fas IgM antibody (1 µg/ml; Panvera, Madison, WI), or 5) TNF-α plus CH-11 antibody (same concentrations). Additional cells from three donors were exposed as above after 1 h of pretreatment with pyrrolidinedithiocarbamate (PDTC, 100 µM), which was used to inhibit nuclear factor (NF)κB activation (17, 18). PDTC was removed before antibody and/or cytokine treatment. As shown in RESULTS, initial studies revealed that CH-11 induced apoptosis in a progressively increasing fraction of cells over 3 days of exposure. Therefore, the influence of the above exposures was assessed on the third day after antibody or cytokine exposure. Because prolonged serum deprivation reduced surface Fas expression (see Fas is expressed on the surface of cultured HASM cells), the influence of antibody and/or cytokine treatment on apoptosis in myocytes deprived of serum for 1 day or for 8 days was evaluated in additional experiments using two coverslips of cells from each of two donors.

Assessment of apoptosis in cultured HASM cells. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) of DNA fragments was used to quantify apoptosis in adherent cultured HASM cells exposed as described in Induction of apoptosis in cultured HASM cells. Stimulated cells were fixed in 1% paraformaldehyde, washed in PBS containing 0.1% Tween 20 (PBS-T), permeabilized in PBS-T containing 1% Triton X-100, and then TUNEL stained with a commercially available kit (Panvera or Boehringer Mannheim, Indianapolis, IN). Nuclei were counterstained with Hoechst 33342 dye or PI. Coverslips were washed in PBS-T and water and mounted onto slides in antifade solution containing glycerol.

Fluorescent staining was assessed with a Nikon microscope equipped with epifluorescence optics and a Photometrics SenSys 12-bit digital video camera; digitized images were recorded with Spectrum imaging software (IP Laboratories, Vienna, VA). The fraction of TUNEL-positive cells was determined by assessing TUNEL staining in 106–1,328 (mean 253) total cells from 6 randomly chosen ×400 fields per condition per coverslip. TUNEL staining was judged positive only when FITC fluorescence was coincident with nuclear staining (with Hoechst 33348 or PI). This requirement excluded occasional FITC-positive debris. TUNEL staining was invariably associated with nuclear pyknosis and/or fragmentation as evident on fluorescent nuclear staining, confirming that in these experiments, positive TUNEL staining reflected apoptotic cell death.

Electrophoretic mobility shift assay of NF-κB activity. Double-stranded DNA oligonucleotides harboring the consensus DNA binding sequence for NF-κB were purchased from Promega (Madison, WI) and were end labeled with T4 polynucleotide kinase and [γ-32P]ATP. Twenty thousand disintegrations per minute (1–5 fmol) were preincubated for 15 min with 1.5 µl of binding buffer (50 mM Tris-HCl, pH 7.5, 20% Ficoll, 357 mM KCl, 5 mM EDTA, and 5 mM dithiothreitol) and 1 µg of poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ). When indicated, a 200-fold molar excess of unlabeled competitor (self or unrelated oligonucleotide containing a consensus myb binding site) was also added. Specific binding was initiated by adding 3 ng of nuclear extract protein obtained from cultured HASM cells with the method of Dignam et al. (7). For supershift experiments, 1–3 µg of antibody were also added to the incubation mixture. Binding reactions were performed at room temperature in a total volume of 15 µl and were continued for 20 min. Nuclear protein complexes were analyzed by electrophoresis on 5% non-denaturing polyacrylamide gels (30:1 acrylamide to bis-acrylamide) in TBE buffer (25 mM Tris borate and 0.5 mM EDTA, pH 8.3) run at 10 V/cm for 1–3 h at 4°C and visualized by autoradiography. Supershift antibodies included anti-p65NFκB (Boehringer Mannheim), anti-p50NFκB (Santa Cruz Biotechnology, Santa Cruz, CA), anti-interleukin-5 (generous gift from Dr. R. Prywes, Columbia University, New York, NY).

RESULTS

Immunohistochemical detection of Fas in HASM. Figure 1 demonstrates that Fas protein is expressed by airway smooth muscle, airway epithelium, and occasional migratory inflammatory cells in a representative human central bronchus. Fas protein was found in a similar distribution in airways from each of the four subjects studied.

Fas is expressed on the surface of cultured HASM cells. Flow cytometry revealed substantial specific fluorescence on live cells stained with anti-Fas DX2 antibody (Fig. 2, inset) before serum deprivation. Serum deprivation for 8 days reduced surface Fas expression partially (P < 0.05 vs. days 0–4 by ANOVA and Tukey’s test; Fig. 2).

Fas cross-linking with CH-11 induces apoptosis in cultured HASM cells, and TNF-α synergistically augments this effect. In an initial study, we examined the time course over which cross-linking of surface Fas with CH-11 antibody induces apoptosis in cultured HASM cells. As shown in Fig. 3, incubation of HASM cells with CH-11 induced apoptosis in a progressively increasing proportion of adherent myocytes over 3 days of exposure. In contrast, exposure to IgM control antibody was without effect. The influence of CH-11 and/or TNF-α exposure on myocytes studied after 1 day of serum deprivation is shown in Fig. 4. Fas cross-linking with CH-11 for 3 days induced apoptosis in a significantly greater fraction (9.7 ± 2.3%) of HASM cells than occurred in untreated (1.1 ± 0.2%) or IgM control treated (0.8 ± 0.1%) cells (P < 0.05 each vs. CH-11 by Friedman ANOVA for repeated measures and Student-
Newman-Keuls multiple comparison analyses). Furthermore, the effect of CH-11 was synergistically augmented by coincubation with TNF-α (31.0 ± 12.2% TUNEL positive; \( P < 0.05 \) vs. CH-11 or TNF-α by Friedman ANOVA and Student-Newman-Keuls), even though TNF-α alone (1.4 ± 0.5%) did not increase TUNEL staining. Importantly, CH-11-induced apoptosis and TNF-α synergy were consistently observed in cells obtained from each of the four donors studied.

Because surface Fas expression was reduced significantly after 8 days of serum deprivation, we tested the effects of antibody and/or cytokine exposure on additional myocytes deprived of serum for 1 day or 8 days. As shown in Fig. 5, right, myocytes deprived of serum for 8 days exhibited no significant apoptosis in response to Fas cross-linking with CH-11 in the absence of TNF-α. However, in marked contrast, myocytes deprived of serum for 8 days treated with both CH-11 and TNF-α exhibited exuberant apoptosis (\( P < 0.05 \) vs. no treatment by ANOVA and Dunnett’s multiple range test), almost at the level of similarly treated myocytes deprived of serum for only 1 day. Note that the 1-day serum-deprived myocytes in this experiment (Fig. 5, left) responded to CH-11, TNF-α, or their combination in a fashion similar to those in the first experiment shown in Fig. 4.

Recent reports (22, 30, 32) have demonstrated that activation of NF-κB ameliorates apoptosis in non-
muscle cells. In addition, Fas ligation can activate NF-κB in nonmuscle cell types (20), and Fas-mediated apoptosis can be potentiated by NF-κB inhibition (34). Based on these findings, we evaluated whether CH-11 treatment in the presence or absence of TNF-α activates NF-κB and whether PDTC pretreatment blunts such activation. Electrophoretic mobility shift assay (EMSA; Fig. 6) revealed that nuclear extracts from cultured HASM cells contain only minimal NF-κB activity, whereas myocytes treated for 75 min with CH-11 or CH-11 plus TNF-α exhibit moderate or exuberant nuclear NF-κB activity (preliminary experiments demonstrated maximal NF-κB activation at 1 h post-stimulation). Pretreatment with PDTC blunted baseline or antibody- and/or cytokine-stimulated NF-κB activity in each circumstance. However, as shown in Table 1, pretreatment with the antioxidant PDTC, which does inhibit NF-κB activity (Fig. 6), had no effect on apoptosis induced by CH-11 in the absence or presence of TNF-α in cultured human airway myocytes. Thus it appears that NF-κB activation does not repress apoptosis induced by Fas cross-linking in these cells.

TNF-α increases surface Fas expression in cultured HASM cells. To explore further the mechanism by which TNF-α increases CH-11-induced apoptosis, we tested the hypothesis that stimulation with TNF-α upregulates surface Fas expression on HASM cells. As shown in Fig. 7, surface Fas expression increased consistently in myocytes exposed to TNF-α for 1–7 days (P ≤ 0.03 for each day by paired t-tests on log-transformed mean specific fluorescence values). Note that TNF-α-induced increases in surface Fas were observed on cells from each donor at each exposure duration studied.

Table 1. Pretreatment with NF-κB inhibitor PDTC had no effect on HASM cell apoptosis induced by CH-11 or CH-11 + TNF-α

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IgM</th>
<th>CH-11</th>
<th>CH-11 + TNF-α</th>
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<tbody>
<tr>
<td>%TUNEL-Positive Cells</td>
<td></td>
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</tr>
<tr>
<td>Nothing</td>
<td>0.8 ± 0.1</td>
<td>9.7 ± 2.3</td>
<td>31.0 ± 12.2</td>
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<tr>
<td>+PDTC</td>
<td>1.1 ± 0.3</td>
<td>12.7 ± 4.2</td>
<td>33.7 ± 21.2</td>
</tr>
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Values are means ± SE. NF, nuclear factor; PDTC, pyrrolidinedithiocarbamate; HASM, human airway smooth muscle; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; TNF, tumor necrosis factor. P values were determined by paired t-tests.

Fig. 4. Incidence of apoptosis in HASM cells stimulated for 3 days with indicated reagents. Values are means ± SE; n = 4 experiments/condition. TNF, tumor necrosis factor. *P < 0.05 vs. all other conditions (by Friedman ANOVA and Student-Newman-Keuls test).

Fig. 5. Incidence of apoptosis in HASM cells stimulated after 1 or 8 days of serum deprivation with indicated reagents. Values are means ± SE. *P < 0.05 vs. respective control (by ANOVA and Dunnett’s test).

Fig. 6. Analysis of nuclear factor (NF)-κB activity in cultured smooth muscle cells with electrophoretic mobility shift assay. Nuclear extract proteins were incubated with radiolabeled oligonucleotide containing a NF-κB consensus binding sequence. NF-κB binding was identified by formation of 2 protein-DNA complexes of reduced electrophoretic mobility (arrows). Specificity of these complexes was established by competition and antibody/supershift experiments, which demonstrated that NF-κB-containing complexes were ablated or substantially reduced by competition with unlabeled self-oligonucleotide or incubation with anti-p65, anti-p50, or both but not by competition with unrelated oligonucleotide (containing an myb binding site) or by incubation with anti-interleukin-5 (IL5) or anti-serum response factor (SRF). Pretreatment with pyrrolidinedithiocarbamate (PDTC) markedly blunts NF-κB activation in control cells (sham stimulated with mouse IgM) and in myocytes stimulated with CH-11 antibody in absence or presence of TNF-α. +, Presence; −, absence.
DISCUSSION

In this study, we provide the first demonstration that Fas protein is expressed by HASM tissue in vivo and on the surface of cultured human airway myocytes in vitro. Furthermore, cross-linking of surface Fas induces apoptosis in a significant number of these cells, an effect that is 1) potentiated by stimulation with TNF-α, which upregulates surface Fas expression, and 2) reduced by prolonged serum deprivation, which, in the absence of TNF-α treatment, reduces surface Fas expression. Our findings confirm and extend previous reports (10, 11), which showed that Fas cross-linking induces apoptosis in TNF-α-treated vascular smooth muscle cells. Thus control of myocyte number through Fas ligation may be a more general mechanism for regulating the architecture of smooth muscle-invested organs.

Immunohistochemical staining for Fas clearly demonstrated its expression within airway smooth muscle in bronchi with normal appearance or minimal inflammation (Fig. 1). This finding documents that Fas is synthesized by bronchial smooth muscle in vivo, but it cannot distinguish whether Fas is expressed at the surface of these cells or whether its ligation might lead to apoptosis. To address these questions, we evaluated Fas surface expression in cultured human airway myocytes in the presence and absence of serum. As shown in Fig. 2, virtually all HASM cells cultured in serum express Fas on their surface, and surface Fas is diminished in myocytes deprived of serum for 8 days. Based on this finding, we tested the hypothesis that Fas cross-linking might induce apoptosis by exposing cultured myocytes to the polyvalent (IgM) CH-11 anti-Fas antibody. In myocytes subjected only to short-term serum deprivation, cross-linking surface Fas did indeed induce apoptosis (Figs. 3 and 4) within 3 days. The fraction of TUNEL-positive cells (10%) approaches that found for other adherent cells undergoing Fas-mediated apoptosis. In contrast, prolonged serum deprivation (8 days), which induces a contractile phenotype (12), reduced surface Fas expression (Fig. 2) and prevented CH-11-mediated killing (Fig. 5). These findings suggest that the phenotypic state of smooth muscle influences its susceptibility to Fas ligation-induced apoptosis, perhaps by regulating surface Fas expression.

Previous reports indicated that TNF-α-treated human vascular smooth muscle cells exhibited increased surface Fas expression (10, 11) and were killed by incubation with Fas cross-linking antibody, whereas non-cytokine-exposed cells were apparently resistant to Fas cross-linking (10). Based on these observations, we tested the influence of TNF-α-treatment on Fas expression and Fas-mediated killing in human airway myocytes. As in vascular smooth muscle, TNF-α exposure increased surface Fas (Fig. 7). Moreover, although TNF-α by itself did not induce substantial apoptosis, it markedly potentiated the killing effect of Fas cross-linking. This potentiating effect of TNF-α was particularly important in 8-day serum-deprived myocytes, in which CH-11 treatment induced apoptosis only in the presence of TNF-α. Together, these findings suggest that TNF-α may exert its effect in part through upregulation of surface Fas, although our study does not exclude other potential mechanisms.

TNF-α has also been reported to ameliorate apoptosis in adherent cells through an NF-κB-dependent mechanism (22, 30, 32). In addition, we (Y. Qin, F. Ko, and K. Hamann, unpublished observations) and others (20) found that Fas ligation can activate NF-κB in nonmuscle cells and that NF-κB inhibition can augment Fas-mediated apoptosis (34). To evaluate whether Fas ligation activates NF-κB in cultured airway smooth muscle cells, we used EMSA to evaluate whether nuclear factors isolated from these cells bound to oligonucleotides containing a consensus NF-κB binding sequence. EMSA revealed that cultured airway myocytes do contain minor NF-κB activity at baseline and that cells treated with CH-11 exhibit further NF-κB activation, especially during coincubation with TNF-α (Fig. 6). To test whether Fas cross-linking-induced NF-κB activation limits apoptosis in CH-11-stimulated airway smooth muscle cells, we stimulated myocytes pretreated with an inhibitor of NF-κB activation. The antioxidant NF-κB inhibitor PDTC (17, 18) had no significant effect on killing induced by Fas ligation with or without concomitant TNF-α exposure (Table 1), even though PDTC did inhibit NF-κB activation as demonstrated by EMSA (Fig. 6). Thus NF-κB does not appear to afford important protection for airway smooth muscle...
against apoptosis under these conditions. Our results in confluent cultured airway myocytes differ from those of Tsai et al. (29), who found that PDTC itself induced apoptosis in exponentially growing aortic myocytes, whereas we found no such effect in airway smooth muscle cells (Table 1). Perhaps the rapidity of growth in their cells, the different tissue of origin, or other experimental features (e.g., their 3- to 12-h vs. our 1-h exposure to PDTC) explain the contrasting effects of PDTC in these two studies. Our results also contrast somewhat with a prior report (2) that serum deprivation induces substantial apoptosis in cultured human aortic smooth muscle. Although 11 days of total serum deprivation (8 days before IgM treatment for an additional 3 days) did increase baseline apoptosis slightly (4.6 ± 0.2% TUNEL positive vs. 2.5 ± 0.5% after 4 days of total serum deprivation (1 day before IgM treatment for 3 more days); P < 0.01 by t-test), this incidence of serum deprivation-induced apoptosis in our airway smooth muscle cells must still be considered small in relation to that stimulated by Fas ligation, especially in the presence of TNF-α (18–31%; Figs. 4 and 5). It is conceivable that our supplementation of serum-free medium with insulin underlies this discrepancy between our study and that of Aoki et al. (2).

The findings reported here suggest a mechanism by which Fas ligand-expressing cells such as T lymphocytes could counterbalance influences that promote airway smooth muscle accumulation in the airways of asthmatic subjects. T lymphocytes are an important migratory inflammatory cell infiltrating asthmatic airways, and T cells are well documented to contact airway smooth muscle. In experimental systems, T cells have already been shown to kill vascular smooth muscle cells through a Fas-dependent mechanism (28), and so it seems reasonable to speculate that T lymphocytes migrating through asthmatic airways might induce apoptosis in bronchial smooth muscle as well. The finding that TNF-α (which is found in asthmatic airways) upregulates surface Fas and Fas-mediated apoptosis further supports this possibility. The potentiating effect of TNF-α might be particularly important in intact tissue in which surface Fas expression might be relatively low, as suggested by our finding of reduced surface Fas as in long-term serum-deprived cultured myocytes (Fig. 2) and substantial potentiation of Fas ligation-induced apoptosis by TNF-α exposure in 8-day serum-deprived cells. Note that even a small incidence of apoptosis might have a significant impact on smooth muscle accumulation within intact asthmatic airways because the proliferative index of airway smooth muscle appears to be small even in the presence of substantial airway inflammation (24).

It is important to consider the limitations of our study. We studied apoptosis in vitro, and it is conceivable that cultured primary myocytes behave differently from airway smooth muscle in situ. In addition, we used an artificial agent, CH-11 antibody, to cross-link and stimulate Fas receptors; perhaps the effects of CH-11 may differ from those of more natural cross-linking with Fas ligand expressed on T lymphocytes.

Finally, cultured passaged airway myocytes tend to modulate toward the synthetic-proliferative phenotype (vs. the contractile phenotype found more typically in intact tissue). We sought to minimize potential effects of modulated smooth muscle phenotype by serum depriving these cells, but it seems likely that phenotypic modulation influences the effectiveness of the Fas-mediated apoptosis pathway (2) as suggested by our results in long-term serum-deprived myocytes.

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