Abrogation of apoptosis through PDGF-BB-induced sulfated glycosaminoglycan synthesis and secretion

Nicholas J. Cartel1,3 and Martin Post1,2,3

1Program in Lung Biology, Research Institute, and Department of Pediatrics, The Hospital for Sick Children, Toronto; and Departments of 2Physiology and 3Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Submitted 22 July 2004; accepted in final form 30 September 2004

Cartel, Nicholas J., and Martin Post. Abrogation of apoptosis through PDGF-BB-induced sulfated glycosaminoglycan synthesis and secretion. Am J Physiol Lung Cell Mol Physiol 288: L285–L293, 2005.—Platelet-derived growth factor (PDGF)-BB-stimulated glycosaminoglycan (GAG) synthesis/secretion in fetal lung fibroblasts is dependent on sequential activation of the PDGF β-receptor, phosphatidylinositol 3-kinase (PI3K), the serine/threonine kinase Akt-1,2, and the GTPase Rab3D. Because the Akt pathway has been implicated in cell survival mechanisms, we investigated whether the pathway regulating GAG synthesis/secretion was antiapoptotic. PDGF-BB treatment protected fetal lung fibroblasts against serum starvation-induced apoptosis, whereas wortmannin, an inhibitor of PI3K, abrogated this protective effect. Transfection of constitutively active Akt into fetal lung fibroblasts also safeguarded the cells from apoptosis induced by serum starvation. To determine whether the antiapoptotic response was due, at least in part, to GAGs, we treated lung fibroblasts with β-d-xylose as well as with topically applied GAGs, specifically those produced by fetal lung fibroblasts. β-d-xylose increased GAG synthesis/secretion and diminished apoptosis. Application of sulfated GAGs, chondroitin sulfate, and heparan sulfate, but not nonsulfated hyaluronan, also resulted in diminished apoptosis. Moreover, topically applied sulfated GAGs increased Bcl-associated death promoter phosphorylation and diminished caspase-3 and -7 cleavage, indicating an antiapoptotic response. These data are compatible with the PDGF-BB-GAG signaling pathway regulating programmed fibroblast death in the fetal lung.

platelet-derived growth factor

APOTOPSIS, OR PROGRAMMED CELL DEATH, IS a cell-autonomous death process requiring cellular enzymatic activity, which does not involve an inflammatory response (27). Numerous mechanisms exist that can either promote or inhibit apoptosis. The orchestration of promotion or inhibition of apoptosis is dependent on the interplay between the various mechanisms that control cell fate during development as well as maintenance of the entire organism in its environment throughout life. Although there are numerous distinct apoptotic and antiapoptotic pathways within a cell, it is evident that there exists communication between the various mechanisms. Communication also exists between extracellular matrix molecules and cell surface receptors as well as by extension, signal transduction cassettes that may include apoptotic and antiapoptotic pathways. Important extracellular matrix molecules are the families of proteoglycans and glycosaminoglycans (GAGs) (19). Proteoglycans consist of specific types of polysaccharide chains, termed GAGs, attached covalently to a core protein. GAGs consist of N-sulfidrylglucosamine or N-acetylglucosamine or N-acetylgalactosamine residues alternating in glycosidic linkages with glucuronic acid, iduronic acid, or galactose residues to form unbranched polysaccharide chains. GAG chains are normally attached to proteoglycans via xylosyl-serine linkages. The GAG constituents of proteoglycans are heparan sulfate, chondroitin/dermatan sulfate, or keratan sulfate. Hyaluronan is not sulfated or linked to a proteoglycan (19). GAGs bind to other matrix components, cell adhesion molecules and growth factors (19, 29), which suggest that GAGs may mediate cell adhesion, branching, differentiation, proliferation, and apoptosis during fetal lung development (30, 32, 33).

It has been demonstrated that cells treated with heparan sulfate upregulate platelet-derived growth factor (PDGF) receptors in lung fibroblasts (25), which can regulate survival and differentiation pathways. Recently, we have shown that PDGF-BB stimulates GAG synthesis by fetal lung fibroblasts (9), which is mediated via the PDGF β-receptor, phosphatidylinositol 3-kinase (PI3K), Akt-1,2, and Rab3D (13, 23). Because fibroblasts have been shown to undergo apoptosis during lung morphogenesis (16, 20) as a means to maximize air-exchange surface area, we investigated the role of PDGF-BB and GAGs in this process. Herein, we demonstrate that sulfated GAGs serve a protective role against apoptosis in fetal rat lung fibroblasts and that the means by which this process occurs is through multiple pathways initiated through the PDGF β-receptor involving PI3K and the serine-threonine kinase Akt.

MATERIALS AND METHODS

Materials. Female (200–250 g) and male (250–300 g) Wistar rats were purchased from Charles River (St. Constant, PQ) and bred in our animal facilities. Rat protocols were in accordance with Canadian Council of Animal Care guidelines and were approved by the Animal Care and Use Committee of the Hospital for Sick Children (Toronto, ON). The sources of all cell culture material have been described elsewhere (10). Sodium [35S]sulfate, [α-32P]ATP, and [3H]NAD were from ICN Biomedicals (St. Laurent, PQ). Human recombinant PDGF-BB was purchased from Upstate Biotechnology (Lake Placid, NY). Protein G-Sepharose, Klenow kinase, and 100-bp ladder DNA marker were purchased from Amersham-Pharmacia Biotech (Baie d’Urfé, PQ). Cytofectin GS was purchased from Glen Research (Sterling, VA). Enhanced green fluorescent protein vector (pEGFP-C1) was purchased from Clontech (Palo Alto, CA). Wortmannin was purchased from Calbiochem (La Jolla, CA). Superspecial grade of skin pig hyaluronic acid (100–1,000 kDa), shark cartilage chondroitin sulfate C (5–40 kDa), and bovine kidney heparan sulfate (2–15 kDa) were purchased from Seikagaku America (Falmouth, MA). p-Nitro-
phenyl-β-D-xylopyranoside (β-D-xyloside), p-nitrophenyl-β-D-galactopyranoside (β-D-galactoside), and sodium chlorate were purchased from Sigma (St. Louis, MO). A kit for TdT-mediated dUTP nick end labeling (TUNEL) was purchased from Roche (Montreal, PQ). Antibodies against full-length and cleaved caspase-3 and -7, as well as antibodies against phosphorylated and unphosphorylated BAD, were purchased from New England Biolabs (Mississauga, ON).

Cell culture. Timed-gestation (day 19, term = 22 days) Wistar rats were killed by diethylether excess, and the fetuses were aseptically removed. The fetal lungs were dissected out, placed in cold Hanks’ balanced salt solution without calcium and magnesium [HBSS(−)], and cleared of major airways and vessels. The lungs were washed twice in HBSS(−), minced, and suspended with HBSS(−). Fibroblasts were isolated from the fetal lungs as previously described (11).

PDGF-BB and GAG stimulation of fetal lung fibroblasts. Fetal rat lung fibroblasts grown to subconfluence in 75-cm² culture flasks were washed three times with serum-free MEM and were serum starved for 24 h at 37°C. The cells were washed again once with serum-free MEM and incubated with either MEM alone or MEM supplemented with reagents for 48 h at 37°C. These reagents included 20 ng/ml PDGF-BB, 25 and 50 mM sodium chlorate, 50 μg/ml β-D-xyloside, 50 μg/ml β-D-galactoside, 100 μM chondroitin sulfate, heparan sulfate, and hyaluronan, respectively.

Wortmannin inhibition of PDGF-BB-stimulated fibroblasts. Fetal lung fibroblasts, washed three times in MEM and serum starved overnight, were preincubated for 30 min in serum-free MEM with or without 500 nM wortmannin at 37°C. Cells were then stimulated with 20 ng/ml of PDGF-BB for 48 h at 37°C.

Transfection of fetal lung fibroblasts. Fetal rat lung fibroblasts (3 × 10⁵ cells/well) seeded into six-well plates were transiently transfected with pcDNA3 plasmids (Invitrogen) containing cDNAs encoding mutated Akt-1 using Cytofectin GS (Glen Research) in MEM plus 5% (vol/vol) FCS. The two plasmids were Akt-DD, which is a constitutively activated form of Akt-1, and Akt-AAA, a dominant negative form of Akt-1 (gifts of Dr. Jim Woodgett, Ontario Cancer Institute, Toronto, ON, Canada). Transfection was allowed to proceed for 6 h before the medium was aspirated and replaced with MEM plus 10% (vol/vol) FCS without rinsing. Medium was replaced 12 h later, and cultures were left undisturbed for another 60 h. Subsequently, cells were washed with serum-free MEM and incubated with either MEM alone or MEM supplemented with 20 ng/ml of PDGF-BB for 48 h at 37°C. Transfection efficiency was determined by transiently transfect-

![Fig. 1. Platelet-derived growth factor (PDGF)-BB prevents serum starvation-induced apoptosis in fetal lung fibroblasts. A: lung fibroblasts were serum starved in MEM alone or MEM + PDGF-BB with and without wortmannin. After 48 h, DNA was isolated, end labeled, and subjected to electrophoresis. B: untransfected fetal lung fibroblasts, under identical conditions as in A, were fixed and processed for TdT-mediated dUTP nick end labeling (TUNEL) staining. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). C: untransfected fetal lung fibroblasts, under identical conditions as in A, were analyzed for poly(ADP-ribose) polymerase (PARP) activity. cpm, Counts per minute.](http://ajplung.physiology.org/)

L286 GLYCOSAMINOGLYCANS ABROGATE APOPTOSIS

AJP-Lung Cell Mol Physiol • VOL 288 • FEBRUARY 2005 • www.ajplung.org

Downloaded from http://ajplung.physiology.org/ by 10.220.33.1 on June 27, 2017
ing fibroblasts with a vector (pEGFP-C1) expressing green fluorescent protein (Clontech). The transfection procedure was performed as aforementioned except that after 24 h, cells were fixed, and nuclei were stained with mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI). The cells were subsequently observed under a fluorescent microscope (Leica Laborlux D, Leitz Wetzlar), and the number of green fluorescent cells was compared with the number of cell nuclei stained with DAPI. The efficiency of transfection was determined to be 75 ± 5% (means ± SE).

**DNA laddering assay.** DNA was extracted from fetal rat lung fibroblasts following treatments using phenol/chloroform. DNA was precipitated from the phenol/chloroform extract using an equal volume of 4 M ammonium acetate, pH 7.5, and two volumes of 95% ethanol. DNA end labeling was performed on 5 μg of purified sample DNA and 2 μg of 100-bp ladder marker DNA. DNA was dissolved in 25 μl of 10 mM Tris·HCl, pH 7.5, and 15 mM MgCl₂. The reaction was initiated by the addition of 10 units of Klenow polymerase and 10 μCi of [32P]dCTP. The samples were incubated for 15 min at 30°C. The reaction was stopped by adding 3 μl of 100 mM EDTA. Labeled DNA was resolved on a 1.2% (wt/vol) agarose gel, which was subsequently dried and exposed to Kodak X-OMAT film.

**TUNEL staining.** Fetal rat lung fibroblasts cultured on glass coverslips (seeded 10⁵ cells/coverlip) and treated according to aforementioned conditions were washed three times in PBS and fixed with a freshly prepared solution of 4% (vol/vol) paraformaldehyde (in PBS, pH 7.4) for 15 min at 4°C. Cells were subsequently rinsed in PBS and permeabilized in a solution containing 0.2% (vol/vol) Triton X-100 and 0.1% sodium citrate for 5 min at 4°C. Slides were processed for TUNEL analysis according to the kit protocol provided by Roche (Montreal, PQ). Cells were embedded, and nuclei were stained with mounting medium containing DAPI. The cells were subsequently observed under a fluorescent microscope (Leica Laborlux D, Wetzel).

![Fig. 2. Transient transfection of fetal lung fibroblasts with a constitutively active Akt prevents serum starvation-induced apoptosis.](image-url)

Lung fibroblasts, transfected with constitutively active Akt-DD or dominant negative Akt-AAA plasmids, were serum starved for 48 h. Untransfected, serum-starved fibroblasts were used as controls. Apoptosis was assessed by DNA laddering (A), TUNEL staining (B), and PARP activity assays (C).
zlar), and the number of green fluorescent cells was compared with
the number of cell nuclei stained with DAPI. Experiments were repeated
at least three times, and five random microscopic viewing frames
per experimental condition were chosen for counting of apoptotic
cells.

Poly(ADP-ribose) polymerase activity measurement. After treat-
ment, poly(ADP-ribose) polymerase (PARP) enzyme activity was
determined from 7.5 × 10⁵ fetal lung fibroblasts, which were
trypsinized and suspended in 5 ml of MEM. The cell suspension was
centrifuged at 1,200 g for 3 min, and the supernatant was discarded.
The pellet was resuspended in 1 ml of buffer A (56 mM HEPES, 28
mM KCl, 28 mM NaCl, and 2 mM MgCl₂). An aliquot (100 µl) of the
cell suspension was mixed in an Eppendorf tube containing 1/10th the
volume of buffer B (2.5 mM NAD, 0.1% digitonin, and 0.3 µCi
[^3]H]NAD) and incubated for 5 min at 37°C. After the incubation, 200
µl of ice-cold 50% trichloroacetic acid (TCA) was added to the
sample, inverted, and left on ice for 10 min, followed by centrifuga-
tion at 10,000 g for 10 min. The supernatant was discarded, and the
pellet was washed twice with ice-cold 20% TCA. The final pellet was
solubilized in 200 µl of buffer C [2% (vol/vol) SDS and 0.1 M
NaOH]. The solubilized sample was incubated overnight at 37°C, and

Immunoblotting. Fetal lung fibroblasts (3 × 10⁵ cells/well in 6-well
culture plates) treated according to aforementioned conditions were
rinsed three times in ice-cold PBS, scraped in lysis buffer [50 mM
HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10%
(vol/vol) glycerol, 1% (vol/vol) Triton X-100, 100 mM sodium
fluoride, 10 mM pyrophosphate, 200 µM sodium orthovanadate, 10
µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF], sonicated,
and centrifuged for 15 min at 10,000 g at 4°C. The protein content
of the supernatant (lysate) was determined according to Bradford (3).
Lysates were boiled for 5 min in sample buffer and subjected to 5%
(wt/vol) SDS-PAGE, transferred to nitrocellulose membrane, and
immunoblotted, in separate experiments, for Bcl-associated death
protein (BAD), caspase-3, and caspase-7 using antibody dilutions of
1:1,000. Nonspecific binding was blocked with 3% (wt/vol) dry milk
powder in PBS. After overnight incubation at 4°C, the membrane was
washed three times with PBS, followed by incubation with horserad-
ish peroxidase-conjugated goat anti-rabbit (1:30,000). After several

Fig. 3. β-D-Xyloside treatment protects fetal
lung fibroblasts against serum starvation-in-
duced apoptosis. Lung fibroblasts were treated
with β-D-xyloside or β-D-galactoside and serum
starved for 48 h. Untreated serum-starved fibro-
blasts were used as controls. Apoptosis was
assessed by DNA laddering (A), TUNEL stain-
ing (B), and PARP activity assays (C).
RESULTS

PDGF-BB treatment is protective against apoptosis. We initially investigated whether there was a protective effect of PDGF-BB against serum starvation-induced apoptosis of fetal rat lung fibroblasts. DNA laddering and TUNEL labeling were used to reveal end-stage apoptotic events, whereas PARP assays (2) were performed to determine cellular response to DNA damage as an early indication of apoptotic activity. DNA from serum-starved fibroblasts as well as serum-starved fibroblasts stimulated with PDGF-BB alone or preincubated with wortmannin and subsequently stimulated with PDGF-BB was isolated, end labeled, electrophoresed, and processed for autoradiography. DNA tandem repeats of 180 bp were detected in the DNA isolated from serum-starved cells and serum-starved cells preincubated with wortmannin and stimulated with PDGF-BB, but not from serum-starved cells stimulated with PDGF-BB alone (Fig. 1A). Inclusion of 10% (vol/vol) FCS with MEM also prevented DNA laddering (data not shown). TUNEL corroborated the DNA laddering findings from the aforementioned conditions. There was a significantly greater amount of TUNEL-positive cells when fibroblasts were serum starved or preincubated with wortmannin and stimulated with PDGF-BB compared with serum-starved fibroblasts stimulated with PDGF-BB alone (Fig. 1B). We further observed a significant increase in PARP activity in fibroblasts that were serum-starved or preincubated with wortmannin and stimulated with PDGF-BB, indicative of cells that have undergone DNA damage, compared with serum-starved cells stimulated with PDGF-BB alone (Fig. 1C). All three assays demonstrate that PDGF-BB stimulation of fetal rat lung fibroblasts is protective against serum starvation-induced apoptosis. Because wortmannin abrogated this protective effect of PDGF-BB on apoptosis, we investigated whether this mechanism of protection is directed through the PDGF-BB-Akt regulatory pathway of GAG synthesis and/or secretion.

Akt is protective against apoptosis. We examined the protective effect of Akt on apoptosis by transiently transfecting constitutively active Akt-DD or dominant negative Akt-AAA into fetal lung fibroblasts. DNA fragmentation was detected in serum-starved cells and serum-starved cells transfected with Akt-AAA, but hardly in serum-starved Akt-DD-transfected cells (Fig. 2A). TUNEL corroborated the DNA laddering findings from the aforementioned conditions (Fig. 2B). We also observed a significant increase in PARP activity in fibroblasts that were serum starved or serum-starved fibroblasts transfected with Akt-AAA compared with serum-starved cells transfected with Akt-DD (Fig. 2C). Together, the data demonstrate that increased Akt activity in fetal lung fibroblasts is protective against apoptosis.

β-D-Xyloside is protective against apoptosis. β-D-Xyloside serves as a proteoglycan core protein substrate for GAG chain elongation and stimulates GAG synthesis of fetal lung fibroblasts (9). Because the PDGF-BB-mediated GAG response is mainly due to GAG chain elongation, sulfation, and secretion (9, 23), we investigated whether fibroblasts treated with β-D-xyloside would be protective against apoptosis induced by serum starvation. We observed that 50 μg/ml of β-D-xyloside, a dosage we have previously shown to stimulate GAG synthesis (9), prevented the DNA laddering compared with control fibroblasts or fibroblasts treated with β-D-galactoside, a carbohydrate control that does not bind GAGs (Fig. 3A). TUNEL also showed that β-D-xyloside treatment was protective against apoptosis (Fig. 3B). Moreover, PARP activity was significantly decreased in fibroblasts treated with β-D-xyloside compared with control or β-D-galactoside-treated fibroblasts (Fig. 3C). These data indicate that β-D-xyloside is an effective antiapoptotic agent for fetal lung fibroblasts.

Sodium chlorate diminishes GAG synthesis. To determine whether PDGF stimulates GAG sulfation, we incubated the cells with sodium chlorate, a global inhibitor of GAG sulfation. Sodium chlorate treatment significantly diminished both basal and PDGF-BB-mediated GAG synthesis from 35SO4, suggesting that sulfate is necessary to mediate PDGF-BB-induced GAG synthesis (Fig. 4). In addition, we found that 25 mM chlorate treatment abolished the PDGF-mediated protection against serum starvation-induced apoptosis as identified by TUNEL analysis (number of TUNEL-positive cell/1,000 cells: control cells 478 ± 59, chlorate-treated cells 584 ± 68, PDGF-BB-treated cells 82 ± 15, PDGF-BB + chlorate-treated cells 395 ± 82; means ± SE, n = 3).

Topical application of sulfated GAGs is protective against apoptosis. Because β-D-xyloside is an artificial substrate for GAG chain elongation, we next investigated whether sulfated or nonsulfated GAGs, topically applied, were protective against apoptosis. Previously, we reported that PDGF-BB upregulated both sulfated and nonsulfated GAGs in fetal lung

Fig. 4. Sodium chlorate treatment diminishes PDGF-BB-mediated glycosaminoglycan (GAG) synthesis. Fetal lung fibroblasts were incubated with 35SO4 and sodium chlorate with or without PDGF-BB. GAGs secreted into the medium were precipitated, and the incorporation of 35SO4 into GAGs was determined. dpm, Disintegrations per minute.
fibroblasts (9); therefore, it would be appropriate to differentiate the effects of GAG species on apoptosis. Heparan sulfate and chondroitin sulfate are two major sulfated GAGs produced by fetal lung fibroblasts (9), which also produce hyaluronan, the only known nonsulfated GAG (9). After topical application of these GAGs on fetal lung fibroblast cultures and subsequent serum starvation for 48 h, isolated DNA from each experiment was used in the DNA laddering assay. Chondroitin sulfate and heparan sulfate, but not hyaluronan, were both protective against apoptosis as demonstrated by the absence of DNA laddering (Fig. 5A). Similarly, fibroblasts treated with hyaluronan displayed significantly greater TUNEL-positive cells (Fig. 5B) and PARP activities (Fig. 5C) compared with fibroblasts treated with chondroitin sulfate or heparan sulfate. Experiments were performed using 50–500 μM of each of the topically applied GAGs. However, data utilizing 100 μM concentrations are presented, as these results already demonstrate significant changes. Hyaluronan was not protective against apoptosis at any of the examined concentrations. These data demonstrate that sulfated GAGs produced by fetal lung fibroblasts can protect them from serum starvation-induced apoptosis.

Topical application of sulfated GAGs results in phosphorylation of BAD and prevents cleavage of caspase-3 and -7. Phosphorylation of BAD leads to its sequestration in the cytosol and contributes early to the inhibition of the apoptotic cascade. Furthermore, prevention of the cleavage of caspase-3 and -7, crucial mediators of late-stage apoptosis, also contributes to the inhibition of the apoptotic cascade. We, therefore, investigated whether topical application of chondroitin sulfate or heparan sulfate stimulated the phosphorylation of BAD and prevented the cleavage of caspase-3 and -7. In contrast to serum-starved cells, treating fibroblasts with PDGF-BB, chondroitin sulfate, or heparan sulfate, but not hyaluronan, led to

Fig. 5. Topically applied heparan sulfate and chondroitin sulfate prevent serum starvation-induced apoptosis in fetal lung fibroblasts. Lung fibroblasts were treated with topically applied hyaluronan, heparan sulfate, or chondroitin sulfate and serum starved for 48 h. Untreated, serum-starved fibroblasts were used as controls. Apoptosis was assessed by DNA laddering (A), TUNEL staining (B), and PARP activity assays (C).
in the PDGF-BB-GAG pathway (23), abrogated this protective
effect. Third, transfection of constitutively active Akt into fetal
lung fibroblasts, which increases GAG synthesis and secretion
without PDGF-BB stimulation (13), safeguarded the fetal lung
fibroblasts from serum starvation-induced apoptosis. Finally,
to determine that the mediator of the antiapoptotic response
was due, at least in part, to GAGs, separate cultures of lung
fibroblasts were treated with β-D-xyloside as well as with
topically applied sulfated GAGs (chondroitin sulfate and hepa-
ran sulfate), both of which resulted in diminished apoptosis.
Moreover, topically applied sulfated GAGs led to an increase
in BAD phosphorylation and diminished caspase cleavage,
indicators of an antiapoptotic response.

An effective alveolar-capillary interface is gradually estab-
lished through expansion of the endothelial, epithelial, and air
space compartments followed by resolution of the interstitial
wall, resulting in thinning of the alveolar septa (26). The
resolution of the interstitium has been previously reported to be
the result of apoptosis during the canalicular and saccular
stages of lung development and is completed during the post-
natal alveolar stage (4, 15, 16). Previously, we reported that
fibroblasts differ in their production of GAGs depending on
their proximity to the epithelium (10). Fibroblasts in close
proximity to the epithelium mainly produce and secrete hya-
luronan, whereas more distant fibroblasts, from the pseudo-
glandular stage of lung development, synthesize primarily
heparan sulfate and chondroitin sulfate. However, this same
population of fibroblasts from the canalicular stage of lung
development produces more hyaluronan (10). The shift to
hyaluronan production correlates with resolution of the alve-
olar septal wall and a concomitant increase in apoptosis,
strongly suggestive of a role for GAGs in lung development.

Because PDGF-BB stimulates sulfated GAG synthesis (8,
9), we treated the cells with sodium chlorate, a global sulfate
inhibitor, and observed that chlorate significantly reduced
PDGF-BB-mediated GAG synthesis from radioactive sulfate.
Moreover, we found that PDGF-BB in the presence of chlorate
did not prevent serum starvation-induced apoptosis, suggesting
the degree of GAG sulfation may play a role in the antiapop-
totic action of PDGF-BB, which fits with the observation that
only sulfated GAGs were protective against apoptosis. The
aglycone derivative of β-D-xyloside used in the current study
was p-nitrophenyl, which is known to prime mainly chon-
droitin/dermatan sulfate (24). Thus the protective effect of
β-D-xyloside against apoptosis likely involves chondroitin sul-
fate, although we cannot exclude dermatan sulfate since we did
not test it individually. Recent studies have shown a wide
distribution of chondroitin sulfates and chondroitin sulfate
proteoglycans in growing fetal rat lung tissue (34), implicating
that these sulfated molecules may play a role in controlling cell
proliferation and cell death. Sulfated GAGs have been shown
to strongly bind growth factors (28, 29). Signaling by the
fibroblast growth factor (FGF) to its high-affinity growth re-
ceptor requires cellular GAGs (36). Moreover, genetic evi-
dence has also demonstrated that FGF signaling requires GAGs
since mutations of genes involved in sulfate activation, sulfate
transport, and GAG synthesis (21, 22, 31) have phenotypes
similar to those of FGF-related gene mutations. Furthermore, it
has been demonstrated that increasingly larger sulfated oligo-
saccharide molecules lead to a greater enhancement of hepa-
tocyte growth factor activation of its cognate receptor c-Met
(38). It has also been shown that heparan sulfate can bind

Fig. 6. Topical application of heparan sulfate and chondroitin sulfate results in
phosphorylation of Bcl-associated death protein (BAD) and prevents caspase
cleavage. Fetal lung fibroblasts were serum starved in the absence or presence
of either PDGF-BB, chondroitin sulfate, heparan sulfate, or hyaluronan, and
phosphorylation of BAD at both Ser<sup>112</sup> and Ser<sup>136</sup> (A) and cleavage of both
caspase-3 and -7 (B) were determined.

**DISCUSSION**

Herein, we demonstrate that the PDGF-BB-GAG signaling
pathway prevents programmed cell death of fetal lung fibro-
blasts. First, we demonstrate that PDGF-BB, which increases
GAG production in fetal lung fibroblasts (9, 13, 23), protected
the cells against serum starvation-induced apoptosis. Second,
wortmannin, an inhibitor of PI3K and a signaling intermediate
in the PDGF-BB-GAG pathway (23), abrogated this protective

**AJP-Lung Cell Mol Physiol • VOL 288 • FEBRUARY 2005 • www.ajplung.org**

Downloaded from http://ajplung.physiology.org/ by 10.220.33.1 on June 27, 2017
PDGF-A and -B chains due to the presence of the trisulfated \( \tau \)-iduronic acid disaccharide unit on its GAG side chain (18). In our culture experiments, topical GAGs were not added to the fibroblasts with PDGF-BB. However, late-stage fetal rat lung fibroblasts express the PDGF-B chain and PDGF \( \beta \)-receptor genes (5, 7). Thus it is possible that the secreted PDGF-BB, albeit in low concentration, can be bound by the sulfated GAGs and presented to the PDGF \( \beta \)-receptor, potentiating the ligand effect, which would be otherwise submaximal. In this manner, it may be possible to activate the receptor and the downstream mechanisms necessary for cell survival. Because it has been determined that complexes between FGF and heparan sulfate are resistant to both heat denaturation and proteolytic degradation (6), it remains possible that PDGF-BB from fetal lung tissue was carried over during the cell culture procedure, further potentiating the effect of the ligand-GAG complex on the PDGF \( \beta \)-receptor.

It has been demonstrated that heparan sulfate upregulates PDGF receptors in lung fibroblasts by threefold over basal expression but has no effect on endogenous expression of PDGF-AA or PDGF-BB (25). This finding in conjunction with the stabilization and concentration of extracellular PDGF-BB by sulfated GAGs, albeit at a low concentration, strengthens the concept that topically applied sulfated GAGs can safeguard a cell from apoptosis by activating signaling mechanisms downstream of the PDGF \( \beta \)-receptor. Therefore, a positive feedback loop reinforces the antiapoptotic signaling mechanism initiated by the application of the sulfated GAGs.

PDGF-BB stimulation of the PDGF \( \beta \)-receptor is an absolute requirement for GAG synthesis to occur (9, 13, 23). Downstream signaling pathways that mediate GAG synthesis and are activated subsequently to PDGF \( \beta \)-receptor transphosphorylation include PI3K and Akt-1,2 (13, 23). We have also demonstrated that p42 MAP kinase (Erk2) is activated subsequent to PDGF-BB stimulation of fetal lung fibroblasts, albeit independently of PDGF \( \beta \)-receptor transphosphorylation (12). Both Akt-1 and Erk1 may have antiapoptotic effects by interfering with BAD. BAD promotes cell death by heterodimerizing with Bcl-2 and Bcl-X\(_L\) in the mitochondria and by contributing to the induction of Bax oligomerization, translocation, and insertion into the mitochondrial membrane that stimulates cytochrome \( c \) release (1, 35). Activated Akt-1 has been shown to bind and phosphorylate BAD at Ser\(^{136} \) (14), whereas activated Erk1 and/or Erk2 phosphorylate BAD at Ser\(^{112} \) (17). Phosphorylation of either site has been demonstrated to prevent BAD from heterodimerizing with Bcl-2 and Bcl-X\(_L\). However, phosphorylation of both sites in BAD results in its binding to the 14-3-3 protein isoforms and subsequent sequestration in the cytosol where it can no longer engage in apoptotic activity (37). It is evident that multiple signaling pathways can be potentially initiated by PDGF-BB and GAGs that protect the cell from apoptosis. Whether the pathways that are activated by sulfated GAGs and PDGF-BB are identical to those activated by PDGF-BB alone remains to be investigated as well as the cross talk between the pathways responsible for apoptotic and antiapoptotic mechanisms.

ACKNOWLEDGMENTS

The authors thank Dr. Jim Woodgett, Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada, for Akt constitutively active and Akt dominant negative plasmids. M. Post holds the Canadian Research Chair in Fetal, Neonatal, and Maternal Health.

GRANTS

This research was supported by Canadian Institutes of Health Research (CIHR) Grant FRN-15273 (to M. Post). N. J. Cartel is a recipient of a Doctoral Research Award from the CIHR.

REFERENCES

23. Liu J, Fitzi D, Liu M, Tseu I, Caniggia I, Rotin D, and Post M. Platelet-derived growth factor-induced glycosaminoglycan synthesis is


