Published in final edited form as:

Biotech Histochem. 2009 December ; 84(6): 287–294. doi:10.3109/10520290903116884.

Exogenous thymosin β_4 prevents apoptosis in human intervertebral annulus cells in vitro

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Abstract

Loss of cells in the human disc due to programmed cell death (apoptosis) is a major factor in the aging and degenerating human intervertebral disc. Our objective here was to determine if thymosin β_4 (TB4), a small, multifunctional 5 kDa protein with diverse activities, might block apoptosis in human annulus cells cultured in monolayer or three-dimensional (3D) culture. Apoptosis was induced in vitro using hydrogen peroxide or serum starvation. Annulus cells were processed for identification of apoptotic cells using the TUNEL method. The percentage of apoptotic cells was determined by cell counts. Annulus cells also were treated with TB4 for determination of proliferation, and proteoglycan production was assessed using cell titer and 1,2 dimethylmethanamine (DMB) assays and histological staining. A significant reduction in disc cell apoptosis occurred after TB4 treatment. The percentage of cells undergoing apoptosis decreased significantly in TB4 treated cells in both apoptosis induction designs. TB4 exposure did not alter proteoglycan production as assessed by either DMB measurement or histological staining. Our results indicate the need for further studies of the anti-apoptotic effect of TB4 and suggest that TB4 may have therapeutic application in future biological therapies for disc degeneration.

Keywords

annulus; apoptosis; cell culture; disc degeneration; thymosin β_4

The objective of our study was to investigate the anti-apoptotic effect of thymosin β_4 (TB4) on cultured human intervertebral annulus cells in vitro. TB4 is a small, multifunctional 5 kDa protein with diverse activities (Goldstein et al. 2005). It is an abundant intracellular protein that has been studied extensively as a G-actin monomer sequestering protein (Safer et al. 1991, Yarmola et al. 2007) involved in actin skeletal remodeling that allows for cell migration (Bubb 2003). TB4 stimulates migration and survival of cardiomyocytes, endothelial cells and corneal cells, and is an essential paracrine factor for embryonic endothelial progenitor cell-mediated cardioprotection (Bock-Marquette et al. 2004, Bubb 2003, Hinkel et al. 2008). TB4 is implicated in many diverse extracellular functions including signaling during wound healing (Philp et al. 2003, 2006), migration of hair follicle stem cells (Philp et al. 2007), and inhibition of cell proliferation (Bonnet et al. 1996, Leeanansaksiri et al. 2004).

Loss of cells due to programmed cell death (apoptosis) is a major factor in the aging and degenerating human intervertebral disc (Freemont 2009, LeMaitre et al. 2007, Park et al. 2005). The human intervertebral disc contains a small cell population that becomes even further reduced with the processes of aging and degeneration. This puts the remaining cell population at high risk for further decrease in cell function or cell numbers (Gruber et al. 2008). Our work and that of others has shown that apoptosis reduces disc cell numbers during aging and degeneration in vivo (Gruber and Hanley 1998, Wang et al. 2008). Previous studies have shown that selected cytokines, such as insulin-like growth factor-1 or platelet-derived growth factor, can be effective for preventing or ameliorating of apoptosis in human annulus cells in vitro (Gruber et al. 2000a). Recent evidence also shows that TB4 has anti-apoptotic effects in various cells (Choi et al. 2006, 2007, Sosne et al. 2004a). This attribute attracted our attention and led to our study of programmed cell death using cultured human annulus cells.

Materials and methods

Human disc cell studies were performed following approval by our human subjects Institutional Review Board. Data are presented from annulus cells from lumbar discs of nine surgical patients (5 female, 4 male; mean age 42 years, range 25–55 years), Thompson grades II – IV (Thompson et al. 1990).

Cell culture

Disc tissue was transported to the laboratory in Minimal Essential Media (MEM, Gibco, Grand Island, NY) within 30 min of removal and established in culture as described previously (Gruber and Hanley 2000b). Briefly, primary cultures were grown in standard T-25 flasks (Costar, Cambridge, MA) containing 5 ml MEM with 20% fetal bovine serum (MEM20; Life Technologies, Grand Island, NY) with antibiotics added. Cells used in experiments were passages 1–3.

Preparation of recombinant TB4

Recombinant human TB4 was purified as described previously (Yarmola et al. 2000, 2001). The TB4 contained an added C-terminal cysteine. Previous results confirmed that the C-terminal cysteine-modified TB4 and TB4 labeled on the C-terminus with tetramethylrhodamine 5-maleimide have identical actin-binding and extracellular properties as wild-type TB4. The concentration of TB4 was confirmed by amino acid analysis. The stocks of TB4 were kept frozen at -70°C and thawed immediately before use.

Apoptosis detection

The assay for apoptosis detection was performed using the R & D systems (Minneapolis, MN) TACS TdT in situ Apoptosis Detection Kit (TUNEL) with modifications described previously (Gruber et al. 1999). Positive and negative controls were included with each TUNEL assay (Gruber et al. 1999). The positive control was established by treating cells with TACS-Nuclease to generate DNA breaks in every cell. This reagent is included in the kit and is the positive control recommended by the package insert. The negative control was established by eliminating the TdT enzyme from the labeling reaction mix.

Cells were seeded into eight-well Nunc slides (LabTek, Naperville, IL) at a density of 5,000 cells/well. Cells were fed TB4 (0, 30, 100 or 800 nM in MEM20) for 24 h. Both the serum starvation and hydrogen peroxide methods described below for inducing apoptosis in disc cells were based on methods described for the TB4 induced apoptosis in corneal cells (Ho et al. 2007, Sosne et al. 2004a).

Hydrogen peroxide treatment

After 24 h in TB4, medium was removed and fresh medium (MEM20) containing 50 μ M hydrogen peroxide, was added for 2 h. Medium was removed and replaced with MEM20 and the cells were incubated for 48 h before terminating the experiment. Control treatments of cells exposed to peroxide without TB4 were included in each experiment. Cells were fixed in 10% neutral buffered formalin (NBF) for 10 min, then transferred to 70% ethanol and processed for apoptosis identification.

Serum starvation

After 24 h in TB4, medium was removed and replaced with MEM without serum. Cells were incubated for 48 h before terminating the experiment. Control treatments of cells treated with serum starvation without TB4 were included in each experiment. Cells were fixed as described above.

Quantitative analysis of the percentage of apoptotic cells

At least 200 cells/treatment were counted. The percentage of apoptotic cells was determined by dividing the number of apoptotic cells by the total number of apoptotic and normal cells counted in each well, and multiplying by 100.

Growth of disc cells in a 3D collagen matrix

Sterile Gelfoam[®] (Pharmacia & Upjohn Co., Kalamazoo, MI), an absorbable collagen sponge prepared from purified porcine skin, used previously in our laboratory to grow intervertebral disc cells in 3D culture (Gruber et al. 2006), was employed as a 3D cell scaffold. Annulus cells were suspended in MEM20 at 1×10^7 cells/ml concentration. Ten microliter droplets containing 1×10^5 cells were injected into collagen sponges trimmed into 0.5 cm³ cubes; 1×10^5 cells/0.5 cm³ of collagen sponges previously have been found to be an optimal number for maximum proteoglycan production in collagen sponges (Gruber et al. 2006). Replicate collagen sponges were placed on Costar Transwell Clear Inserts (Corning Incorporated-Life Sciences, Lowell, MA) in 24-well plates and fed three times per week with 2.0 ml of medium (MSCBM) with TB4 (30 nM) or without TB4 (untreated control). The concentration of 30 nM TB4 was chosen based on previous observations of cytokine-like activity of TB4 at low doses in mast cells (Leeanansaksiri et al. 2004). Cells were grown for 2 weeks and assayed for proteoglycan production in the presence or absence of TB4. Cultures were terminated, fixed in 10% NBF for 1 h and embedded in paraffin. Collagen sponges were sectioned for histological analysis and stained for extracellular matrix proteoglycan production using toluidine blue (0.1% in distilled water; Sigma). Proteoglycan production also was assessed quantitatively using the 1,9-dimethylmethylene blue (DMB) assay described below.

Quantitative measurement of total sulfated glycosaminoglycan production

Cells were grown in 3D culture for 14 days in the presence or absence of TB4 (30 nM) and assayed for sulfated proteoglycan production using the DMB assay (Muller and Hanschke 1996). Briefly, collagen sponges containing cells were rinsed once in Hank's Balanced Salt Solution (HBSS). Replicates were placed in 1.5 ml microcentrifuge tubes and collagenase type V (Sigma) in HBSS was added to produce a final concentration of 1.0 Units/ml. Proteoglycan concentrations were determined from the standard curve, replicates averaged, and results expressed as micrograms S-GAG/ ml.

Measurement of cell proliferation

Cell proliferation was evaluated by seeding replicate samples of disc cells, 5,000 cells per well, in monolayer in 48-well tissue culture plates and either treating or not with TB4 (0–800 nM).

After 3 days of culture, 20 μ l of kit reagent was added (Cell Titer 96 Aqueous One Solution Kit; Promega, Madison, WI) (Cory et al. 1991). Plates were wrapped in foil, shaken for 5 min, and incubated for 2 h at 37° C. Absorbance at 490 nm was measured using a plate reader (EL 800 Universal Micro Plate Reader; Bio-Tek Instruments, Inc). Data are expressed as means $\pm$ SD of 16 replicate wells per treatment.

Statistical analysis

Data were analyzed using SAS version 8.2. A p-value < 0.05 was considered statistically significant. Standard statistical methods were used including repeated measure analysis of variance. Data are presented as means $\pm$ SD (n).

Results

Anti-apoptotic effect of TB4 on intervertebral annulus cells

Three days after exposure to the apoptosis inducing agents, human annulus cells induced to undergo apoptosis by hydrogen peroxide exposure showed both normal appearing spindle shaped cells and cells that were rounded (Fig. 1). Most cells under serum starvation conditions continued to be spindle shaped for the duration of the experiment.

Using the TUNEL apoptosis assay, apoptotic cells exhibiting DNA fragmentation were stained brown (Figs. 2 and 3) and were readily distinguished from non-apoptotic or necrotic cells, counterstained green. Positive and negative experimental control cells also are shown in Figs. 2 and 3. TB4 (800 nM) was found to prevent apoptosis induced by both hydrogen peroxide and serum starvation in the disc cells (Figs. 2 and 3).

In our initial studies, we evaluated TB4 in a dose-response experimental design. Preliminary experiments using lower concentrations of TB4 with the hydrogen peroxide treatment (30 and 100 nM) showed no significant reduction in hydrogen peroxide induced disc cell apoptosis ($21.5\% \pm 6.2$ (3) [mean $\pm$ S.D. (n)] in controls vs. $27.4\% \pm 8.2$ (3) in 30 nM TB4 treated cells and $14.2\% \pm 11.6$ (5) in controls vs. $8.7\% \pm 8.3$ (5) in 100 nM TB4-treated cells). A significant reduction in disc cell apoptosis was seen after 800 nM TB4 treatment following either hydrogen peroxide (Fig. 2) or serum starvation (Fig. 3) induced apoptosis.

The percentage of cells undergoing apoptosis decreased significantly in TB4 treated cells for both apoptosis induction designs: hydrogen peroxide induced apoptosis, $46.9\% \pm 10.9$ (5) in controls vs. $21.4\% \pm 6.1$ (5) in TB4 treated cells ($P = 0.0437$) (Fig. 2); serum starvation design, $38.8\% \pm 8.1$ (5) for controls vs. $12.3\% \pm 8.4$ (5) in TB4 treated cells ($P = 0.0347$) (Fig. 3).

Effect of TB4 on annulus cells in 3D culture

TB4 previously has been shown to stimulate production of laminin-5 in the extracellular matrix of human corneal epithelial cells (Sosne et al. 2004b, 2007). TB4 also has been shown to exert a cytokine-like activity at very low doses in mast cells (Leeanansaksiri et al. 2004). Two main classes of extracellular macromolecules make up the extracellular matrix: proteoglycans, and fibrous proteins including collagen and laminin, which have both structural and adhesive functions. To test whether TB4 at very low cytokine-like doses has an effect similar to cancer cells on annulus extracellular matrix production of proteoglycans, we grew the cells in 3D collagen culture and exposed them to 30 nM TB4 for 14 days. Fig. 4A shows the growth of disc cells in 3D collagen culture with or without 30 nM TB4. In 3D cultures, the rounded cells are clearly visible growing in and around the 3D scaffold (Fig. 4A). No significant differences were observed in the appearance of the TB4 treated cells. The 3D cultures were stained with toluidine blue, which stains proteoglycans pink. Slight pink staining is visible around the cells showing the presence of proteoglycans in the extracellular matrix produced by the disc cells

(Fig. 4A). Although in this particular image more cells happen to be present in the TB4 treated cultures, no increase in the amount of proteoglycans around each cell is evident.

The presence of proteoglycans in 3D cultures treated or untreated with TB4 also was confirmed quantitatively using the DMB assay. No significant difference in proteoglycan production was observed when the cells were treated with TB4 (Fig. 4B) ($0.81 \text{ mg/ml} \pm 0.44$ (3) for controls vs. 0.94 ± 0.42 (3) for TB4 treated cells).

TB4 does not affect annulus cell proliferation

TB4 has been shown previously to inhibit cell proliferation in mast cells and hematopoietic stem cells (Bonnet et al. 1996, Leeanansaksiri et al. 2004). To determine the effect of TB4 on disc cell proliferation after exposure to TB4, we performed the cell titer colorimetric assay where light absorption at 490 nm is proportional to the number of cells present (Cory et al. 1991). No significant change in proliferation was observed following exposure to concentrations from 1 to 800 nM ($1.66 \text{ nm} \pm 0.15$ (3) for controls vs. $1.72 \text{ nm} \pm 0.14$ (3) for TB4 treated cells).

Discussion

In the study reported here, we have shown that exposure to 800 nM of exogenous TB4 exerts an anti-apoptotic effect on cultured annulus cells following application of the apoptotic inducing external stresses, hydrogen peroxide exposure or serum starvation. Although TB4 is known to be internalized by cells, the cell surface binding site of TB4 currently is unknown (Bubb 2003). When fibroblastic cells were induced to undergo apoptosis by anti-tumor drugs, intracellular TB4 levels decreased (Iguchi et al. 1999). Increased resistance to apoptosis in cells over-expressing TB4 also has been reported (Niu and Nachmias 2000).

Cell apoptosis is known to involve mechanisms such as activation of caspases-3/7, which are mediated extrinsically by common apoptosis inducing factors such as FasL, tumor necrosis factor alpha (TNF- α), and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Zhang et al. 2008) or by intrinsic mitochondrial pathways such as mitochondrial involvement in Fas-mediated apoptosis in disc cells (Park et al. 2005). In corneal cells, TB4 has been shown to function as an anti-apoptotic agent by inhibiting the release of cytochrome c from mitochondria and by suppressing the activation of caspases (Sosne et al. 2004a). Although the receptors involved in binding TB4 to cells are unknown, internalization is essential for the anti-apoptotic effects of TB4 (Ho et al. 2007). To date, the anti-apoptotic effect of TB4 on human disc cells has been unexplored.

Modulation of apoptosis with cytokines and other reagents is a potential therapeutic strategy for treating disc degeneration (Zhao et al. 2006). Degenerated discs have decreased proteoglycan content associated with loss of load bearing function. During early stages of disc degeneration, stimulation of matrix synthesis by disc cells may be feasible using a number of potential biological therapies (Gruber and Hanley 2003). TB4 has been implicated in wound healing and increased production of laminin-5, an important protein for cell-extracellular matrix adhesion, in the extracellular matrix of human corneal epithelial cells (Sosne et al. 2004b, 2007). TB4 has been found to induce both TGF β and laminin-5 gamma 2 chain expression in corneal epithelial cells (Sosne et al. 2004b).

In our study, no change in proteoglycans in the extracellular matrix produced by disc cells exposed to low doses of TB4 was detectable by toluidine blue staining or quantitative proteoglycan assay. It is possible that future studies of synergistic interactions of TB4 with cytokines such as TGF β may show stimulation of matrix production.

TB4 has been found to have varying effects on proliferation of different types of cells. The presence of TB4 did not effect the proliferation of corneal cells (Sosne et al. 2006), but has been shown previously to inhibit proliferation of hematopoietic progenitor cells and mast cells (Bonnet et al. 1996, Leraanansaksiri et al. 2004). We were unable to identify a positive effect of TB4 on disc cell proliferation.

Further studies of the effects of TB4 on cell survival and proliferation and of diverse cell processes along different cellular pathways (Sosne et al. 2004a) are warranted, as is further investigation into the mechanisms by which TB4 inhibits apoptosis in annulus cells.

Our work has shown that TB4 suppressed or prevented apoptosis in human intervertebral annulus cells following apoptotic stresses in vitro; however, TB4 did not influence cell proliferation or alter proteoglycan production by annulus cells. Our results suggest that TB4, possibly administered by injection to specific disc sites, may have future therapeutic potential for biological therapies for disc degeneration.

Acknowledgments

We gratefully acknowledge the technical assistance of Dr. Jim Norton and Ms Natalia Zinchenko. This work was funded by a grant from the Charlotte-Mecklenburg Health Services Foundation, the Carolinas Back Pain Research Endowment, the Medical Research Service of the Department of Veterans Affairs, the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), grant number 5K25AR048918 to EGY and the National Science Foundation grant number NSF-0316015 to MRB.

References

- Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature* 2004;432:466–472. [PubMed: 15565145]
- Bonnet D, Lemoine FM, Frobert Y, Bonnet ML, Baillou C, Najman A, Guigon M. Thymosin beta4, inhibitor for normal hematopoietic progenitor cells. *Exp. Hematol* 1996;24:776–782. [PubMed: 8647227]
- Bubb MR. Thymosin beta 4 interactions. *Vitam. Horm* 2003;66:297–316. [PubMed: 12852258]
- Choi SY, Kim DK, Eun B, Kim K, Sun W, Kim H. Anti-apoptotic function of thymosin-beta in developing chick spinal motor neurons. *Biochem. Biophys. Res. Commun* 2006;346:872–878. [PubMed: 16782066]
- Choi SY, Noh MR, Kim DK, Sun W, Kim H. Neuroprotective function of thymosin-beta and its derivative peptides on the programmed cell death of chick and rat neurons. *Biochem. Biophys. Res. Commun* 2007;362:587–593. [PubMed: 17716628]
- Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991;3:207–212. [PubMed: 1867954]
- Freemont AJ. The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. *Rheumatology* 2009;48:5–10. [PubMed: 18854342]
- Goldstein AL, Hannappel E, Kleinman HK. Thymosin beta4: actin-sequestering protein moonlights to repair injured tissues. *Trends Mol. Med* 2005;11:421–429. [PubMed: 16099219]
- Gruber HE, Hanley EN Jr. Analysis of aging and degeneration of the human intervertebral disc. Comparison of surgical specimens with normal controls. *Spine* 1998;23:751–757. [PubMed: 9563104]
- Gruber HE, Norton HJ, Hanley EN Jr. Anti-apoptotic effects of IGF-1 and PDGF on human intervertebral disc cells in vitro. *Spine* 2000a;25:2153–2157. [PubMed: 10973395]
- Gruber HE, Hanley EN Jr. Human disc cells in monolayer vs. 3D culture: cell shape, division and matrix formation. *BMC Musculoskel. Disord* 2000b;1:1.
- Gruber HE, Hanley EN Jr. Biologic strategies for the therapy of intervertebral disc degeneration. *Exp. Opin. Biol. Ther* 2003;3:1209–1214.

- Gruber HE, Coleman SS, Stasky AA, Hoelscher GL, Desai BJ. Detection of apoptotic cells in vitro: a practical standardized experimental protocol using human fibroblasts for initial laboratory studies. *Biotech. & Histochem* 1999;74:141–145.
- Gruber HE, Hoelscher GL, Leslie K, Ingram JA, Hanley EN Jr. Three-dimensional culture of human disc cells within agarose or a collagen sponge: assessment of proteoglycan production. *Biomaterials* 2006;27:371–376. [PubMed: 16098581]
- Gruber HE, Ingram JA, Davis DE, Hanley EN Jr. Increased cell senescence is associated with decreased cell proliferation in vivo in the degenerating human annulus. *Spine J.* 2008
- Hinkel R, El-Aouni C, Olson T, Horstkotte J, Mayer S, Müller S, Willhauck M, Spitzweg C, Gildehaus FJ, Münzing W, Hannappel E, Bock-Marquette I, DiMaio JM, Hatzopoulos AK, Boekstegers P, Kupatt C. Thymosin beta4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. *Circulation* 2008;117:2232–2240. [PubMed: 18427126]
- Ho JH, Chuang CH, Ho CY, Shih YR, Lee OK, Su Y. Internalization is essential for the antiapoptotic effects of exogenous thymosin beta-4 on human corneal epithelial cells. *Invest. Ophthalmol. Vis. Sci* 2007;48:27–33. [PubMed: 17197512]
- Iguchi K, Usami Y, Hirano K, Hamatake M, Shibata M, Ishida R. Decreased thymosin beta4 in apoptosis induced by a variety of antitumor drugs. *Biochem. Pharmacol* 1999;57:1105–1111. [PubMed: 11230797]
- Leeanansaksiri W, DeSimone SK, Huff T, Hannappel E, Huff TF. Thymosin Beta4 and Its N-Terminal Tetrapeptide, AcSDKP, Inhibit Proliferation, and Induce Dysplastic, Non-Apoptotic Nuclei and Degranulation of Mast Cells. *Chem. Biodivers* 2004;1:1091–1100. [PubMed: 17191900]
- LeMaitre CL, Pockett A, Buttle DJ, Freemont AJ, Hoyland JA. Matrix synthesis and degradation in human intervertebral disc degeneration. *Biochem. Soc. Trans* 2007;35:652–655. [PubMed: 17635113]
- Muller G, Hanschke M. Quantitative and qualitative analyses of proteoglycans in cartilage extracts by precipitation with 1,9-dimethylmethylene blue. *Connec. Tissue Res* 1996;33:243–248.
- Niu M, Nachmias VT. Increased resistance to apoptosis in cells over expressing thymosin beta four: A role for focal adhesion kinase pp125FAK. *Cell Adhes. Commun* 2000;7:311–320. [PubMed: 10714392]
- Park J-B, Lee J-K, Park S-J, Kim K-W, Riew KD. Mitochondrial involvement in Fas-mediated apoptosis of human lumbar disc cells. *Am. J. Bone Joint Surg* 2005;87A:1338–1342.
- Philp D, Huff T, Gho YS, Hannappel E, Kleinman HK. The actin binding site on thymosin beta4 promotes angiogenesis. *FASEB J* 2003;17:2103–2105. [PubMed: 14500546]
- Philp D, Scheremeta B, Sibbliss K, Zhou M, Fine EL, Nguyen M, Wahl L, Hoffman MP, Kleinman HK. Thymosin beta4 promotes matrix metalloproteinase expression during wound repair. *J. Cell. Physiol* 2006;208:195–200. [PubMed: 16607611]
- Philp D, St-Surin S, Cha HJ, Moon HS, Kleinman HK, Elkin M. Thymosin beta 4 induces hair growth via stem cell migration and differentiation. *Ann. N.Y. Acad. Sci* 2007;1112:95–103. [PubMed: 17947589]
- Safer D, Elzinga M, Nachmias VT. Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. *J. Biol. Chem* 1991;266:4029–4032. [PubMed: 1999398]
- Sosne G, Albeiruti AR, Hollis B, Siddiqi A, Ellenberg D, Kurpakus-Wheater M. Thymosin beta4 inhibits benzalkonium chloride-mediated apoptosis in corneal and conjunctival epithelial cells in vitro. *Exp. Eye Res* 2006;83:502–507. [PubMed: 16630613]
- Sosne G, Siddiqi A, Kurpakus-Wheater M. Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. *Invest. Ophthalmol. Vis. Sci* 2004a;45:1095–1100. [PubMed: 15037574]
- Sosne G, Xu L, Prach L, Mrock LK, Kleinman HK, Letterio JJ, Hazlett LD, Kurpakus-Wheater M. Thymosin beta 4 stimulates laminin-5 production independent of TGF-beta. *Exp. Cell Res* 2004b;293:175–183. [PubMed: 14729067]
- Sosne G, Qiu P, Christopherson PL, Wheeler MK. Thymosin beta 4 suppression of corneal NFkappaB: a potential anti-inflammatory pathway. *Exp. Eye Res* 2007;84:663–669. [PubMed: 17254567]
- Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IK, Bishop PB. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. *Spine* 1990;15:411–415. [PubMed: 2363069]

- Wang D, Liu M, Song H, Wang M, Yang K, Zhang Y. Expression of Bax and Caspase-3 and apoptosis in human lumbar intervertebral disc degeneration. *Chung-Kuo Hsiu Fu Chung Chien Wai Ko Tsa Chih/Chinese Journal of Reparative & Reconstructive Surgery* [Q2] 2008;22:421–425.
- Yarmola EG, Somasundaram T, Boring TA, Spector I, Bubb MR. Actin-Latrunculin A Structure and Function: Differential modulation of actin-binding protein function by latrunculin A. *J. Biol. Chem* 2000;275:28120–28127. [PubMed: 10859320]
- Yarmola EG, Parikh S, Bubb MR. Formation and implications of a ternary complex of profilin, thymosin beta-4, and actin. *J. Biol. Chem* 2001;276:45555–45563. [PubMed: 11579089]
- Yarmola EG, Klimenko ES, Fujita G, Bubb MR. Thymosin beta4: actin regulation and more. *Ann. N.Y. Acad. Sci* 2007;1112:76–85. [PubMed: 17947588]
- Zhang L, Niu T, Yang SY, Lu Z, Chen B. The occurrence and regional distribution of DR4 on herniated disc cells: a potential apoptosis pathway in lumbar intervertebral disc. *Spine* 2008;33:422–427. [PubMed: 18277875]
- Zhao CQ, Jiang LS, Dai LY. Programmed cell death in intervertebral disc degeneration. *Apoptosis* 2006;11:2079–2088. [PubMed: 17051327]

Fig. 1. Cultured human annulus cells 3 days after serum starvation or hydrogen peroxide treatment prior to fixation for apoptosis assay. A) Annulus cells untreated or treated with 800 nM TB4 previously exposed to serum starvation. B) Annulus cells treated or untreated with 800 nM TB4 exposed to hydrogen peroxide. Most cells show typical spindle shaped morphology. Some rounded, shrunken, cells also were present in the hydrogen peroxide treated cells. $\times 115$.

Fig. 2.

Anti-apoptotic effect of 800 nM TB4 on annulus cells. Annulus cells with or without TB4 treatment after induction of apoptosis by hydrogen peroxide. Apoptotic cells are stained brown and non-apoptotic cells are counterstained green. Positive and negative experimental controls also are shown. $\times 95$. Graph shows percent of apoptotic cells in the treated and untreated cultures. Data from five TUNEL analyses were analyzed using repeated measure analysis of variance; data were significant, $*p < 0.05$.

Fig. 3.

Anti-apoptotic effect of 800 nM TB4 on annulus cells. Annulus cells with or without TB4 treatment after induction of apoptosis with serum starvation. Apoptotic cells are stained brown and non-apoptotic cells are counterstained green. Positive and negative experimental controls are also shown. $\times 95$. Graph shows percent of apoptotic cells in the treated and untreated cultures. Data from five TUNEL analyses were analyzed using repeated measure analysis of variance; data were significant, $*p < 0.05$.

Fig. 4.

Effect of 30 nM TB4 on annulus cells in 3D culture. After 2 weeks in 3D culture, both untreated and TB4 treated annulus cells appeared healthy with the typical rounded morphology of annulus cells in 3D culture. Slight pink staining shows the presence of proteoglycans in the extracellular matrix produced by the annulus cells. $\times 95$. Graph shows DMB analysis from three separate cultures. Statistical analysis using repeated measure analysis of variance showed no change in proteoglycan production with or without TB4.