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Regulation of human nucleus pulposus cells by peptide-coupled substrates

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ABSTRACT

Nucleus pulposus (NP) cells are derived from the notochord and differ from neighboring cells of the intervertebral disc in phenotypic marker expression and morphology. Adult human NP cells lose this phenotype and morphology with age in a pattern that contributes to progressive disc degeneration and pathology. Select laminin-mimetic peptide ligands and substrate stiffnesses were examined for their ability to regulate human NP cell phenotype and biosynthesis through the expression of NP-specific markers aggrecan, N-cadherin, collagen types I and II, and GLUT1. Peptide-conjugated substrates demonstrated an ability to promote expression of healthy NP-specific markers, as well as increased biosynthetic activity. We show an ability to re-express markers of the juvenile NP cell and morphology through control of peptide presentation and stiffness on well-characterized polyacrylamide substrates. NP cells cultured on surfaces conjugated with α3 integrin receptor peptides P4 and P678, and on α2, α5, α6, β1 integrin-recognizing peptide AG10, show increased expression of aggrecan, N-cadherin, and types I and II collagen, suggesting a healthier, more juvenile-like phenotype. Multi-cell cluster formation was also observed to be more prominent on peptide-conjugated substrates. These findings indicate a critical role for cell-matrix interactions with specific ECM-mimetic peptides in supporting and maintaining a healthy NP cell phenotype and bioactivity.

Statement of Significance

NP cells reside in a laminin-rich environment that deteriorates with age, including a loss of water content and changes in the extracellular matrix (ECM) structure that may lead to the development of a degenerated IVD. There is great interest in methods to re-express healthy, biosynthetically active NP cells using laminin-derived biomimetic peptides toward the goal of using autologous cell sources for tissue regeneration. Here, we describe a novel study utilizing several laminin mimetic peptides conjugated to polyacrylamide gels that are able to support an immature, healthy NP phenotype after culture on "soft" peptide gels. These findings can support future studies in tissue regeneration where cells may be directed to a desired regenerative phenotype using niche-specific ECM peptides.

1. Introduction

Nucleus pulposus (NP) cells are derived from the embryonic notochord and are responsible for the original synthesis and maintenance of the extracellular matrix (ECM) of the intervertebral disc. An early decrease in their cell number, loss of this developmental phenotype, and infiltration of alternate cell types are considered critical events in the alterations in mechanical function associated with intervertebral disc degeneration [1–3]. NP cells may interact with collagens, fibronectin and laminins of the extracellular matrix (ECM) through integrin and non-integrin mediated mechanisms [4–10] with profound effects on cellular biosynthesis, attachment and morphology. Studies have shown the α5β1 integrin heterodimer regulates NP cell interactions with fibronectin [8], and are also

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involved in the onset of cell pathobiology following exposure to degraded fragments of fibronectin [11]. Studies of rat NP cells have shown that attachment to type II collagen is mediated by the α2 integrin subunit in a process that involves activation of extracellular signal-regulated kinase-1 (ERK) [5], while porcine NP cells were instead shown to use the α1 integrin subunit to attach to type II collagen [8]. While collagens and fibronectin are compositionally abundant in the intervertebral disc, NP cell interactions with laminin proteins may be a key feature that distinguishes juvenile from aged, degenerate NP cells. Multiple isoforms of laminin are present in the juvenile NP, but not adjacent anulus fibrosus (AF) regions, as identified by immunohistochemical staining for the γ1 and other laminin chains [9,12]. Porcine NP cells have been shown to interact with laminins LM-111 and LM-511 through integrins α6 and β1 [8,13], while human NP cells derived from aged, degenerate tissues rely upon integrins α3, α5 and β1 for binding to these same laminins [10]. Together, these findings begin to reveal a role for specific integrin subunits in mediating NP cell-ECM interactions.

Peptides derived from ECM molecules may act as cell recognition motifs and can be used to increase cell attachment and elicit specific cell responses [14]. As compared to full-length proteins, peptides have many beneficial properties, such as receptor specificity, increased stability, ease of coupling, and cost effectiveness. The most commonly used cell recognition peptide, RGD (Arg-Gly-Asp), was derived from fibronectin [15] and has been shown to interact with integrins α5β1 and αVβ5, and up to 12 additional integrin subunits [16–18]. Of relevance to NP cells, a mechanically stimuli-driven increase in ECM production for NP cells can be attenuated when incubating cells with the RGD peptide, given evidence of functional interactions for NP cells with RGD [7]. To date, there is no information on peptide effects on NP cells other than RGD. In our prior work, we have demonstrated that surfaces coupled with full-length laminins (LM-111, LM-511), or basement membrane extract rich in laminins, can promote healthier, more biosynthetically active NP cells. These cells cultured on laminin-presenting substrates show elevated glycosaminoglycan (GAG) synthesis, a prototypical rounded and clustered cellular morphology, and elevated expression of healthy NP molecular markers including N-cadherin, type II collagen, and brachyury [13,19–21]. In particular, we have identified laminin-coupled substrates of polyethylene glycol (PEG) or polyacrylamide (PAAm) that are uniquely well-suited to promote these features when engineered with stiffness less than 0.5 kPa [21–23]. Thus, cell recognition peptides derived from laminins may play a unique role in regulating NP cell interactions and behaviors. Nomizu and co-workers have been screening laminin chains since 1995 for active peptide sequences, resulting in a library of laminin-derived bioactive peptides [24–27]. Due to these and other similar studies, there is a large selection of laminin-derived peptides that have been shown to engage specific integrin and non-integrin cell surface receptors and to modulate the behaviors of multiple cell types (e.g. [28–31]). The objective of this study was to identify a subset of ECM mimetic peptides that can regulate human NP cell attachment, morphology and behaviors, and to reveal if peptide-coupled substrates of varying stiffness and peptide specificity can maintain the healthy NP-specific cell phenotype.

2. Materials and methods

2.1. Primary human NP cell culture

Cells from the NP region of to-be-discarded surgical waste tissues (ages 33–77, degenerative scoliosis, non-human subjects, IRB exemption) from lumbar and thoracic regions were isolated using a pronase-collagenase digestion, as previously described [10]. Cells from each patient were collected as separate samples, but vertebral level and grade of pathology were not known for the non-human subjects research designation (only age, race and gender information were collected). Briefly, NP tissue was separated from the surgical sample and surrounding annulus fibrosus and cartilaginous tissue and digested 2–4 h in 25 mL digestion solution per gram of NP tissue (0.3% collagenase type II, 0.2% pronase). The cells were expanded up to passage 2 in F-12 media supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin under 5% CO_2.

2.2. Peptide selection for NP cell attachment

Ten ECM mimetic peptides were chosen for their demonstrated role in regulating cell adhesion and spreading in other cell types [29,31–40]. Peptide selection was additionally constrained by using peptides derived from laminins and/or peptides shown to engage integrin α3, α5, or α6 (Table 1), based on demonstrated interactions of NP cells with laminin through these integrin subunits. The final set of peptides were synthesized with a cysteine-glycine-glycine at the N-terminus (American Peptide Co., Sunnyvale, CA). The peptides were manufactured at over 95% purity and solubilized in ultrapure water at 1 mg/mL; peptides p678 and AG10 were the exception, requiring 20% acetonitrile to fully solubilize at 1 mg/mL. Stock peptides were stored at −80 °C until use. Experimental peptide solutions were prepared from the stock by dilution with ultrapure water.

2.3. NP cell attachment to peptide coated surfaces

Substrates (96 half-well assay plate, Corning) were prepared by adding 40 μl of each peptide solution to each well at 0.1, 1, 10, 100, and 200 μg/mL on the plates; controls included wells with no protein (PBS only), mouse LM-111 at 20 μg/mL (Millipore, Billerica, MA), or mouse LM-511 at 10 μg/mL (Sigma-Aldrich) (all values chosen based on prior work showing maximal NP cell attachment [8,10]. Plates were incubated overnight at 4 °C to adsorb the peptides to the surface of the plastic. The wells were then blocked with 3.75% bovine serum albumin (BSA) for 3 h at 37 °C followed by one rinse with 1X PBS. Wells without protein but blocked with BSA were negative controls to identify non-specific cell binding.

NP cells (tissue ages 30–77, n = 9 patients) were detached from tissue culture plastic, seeded in serum-free media in protein-coated wells (4000 cells/well), allowed to attach for 1 h, and washed twice with serum-free media to remove unattached cells. Attached cells were lysed and counted using the CellTiter-Glo

Table 1
Peptides selected for screening against NP cells.

Peptide name	Amino acid sequence	Reported cell receptors
GD6	CGG(KQNCLSSRAS) FRGCVRNLRLSR	Integrin α3 [32,35,40,44,45]
P4	CGGPPFLMLLKGSTR	Integrin α3 [32,35,44,45,71]
p678	CGGFQGVLQNVRFVF	Integrin α3 [32,34,35,44,45,71]
cNGQ	CGGcMGQGEQc (cyclic)	Integrin α3 [32,35,44,45]
AG32	CGGTWYKIAFQRNRK	Integrins α2, α6, β1 [27,29,38]
AG10	CGGNRWHSIYITRFG	Integrins α2, α5, α6, β1 [27,29,38]
T1	CGGTTSWSQCSKS	Integrin α6 [31,46,47]
AG73	CGGRKRLQVQLSIRT	Syndecans [25,27,37,49,53–56,72]
RGD	CGGRGDS	Integrins α5, αV, α8 [15–18,50,51]
IKVAV	CSRARKQAASIKVAVSADR	Integrins [33,39,48,49]

Laminin-mimetic peptides used for initial screening.