ABSTRACT
Objective: To investigate the effects of proteins products of endothelial cells (ECs) on the annulus fibrosus (AF) cell metabolism in an in vitro culture. Methods: Human AF cells were expanded in monolayer cultures and treated with proteins from the medium of cell line HMEC-1 (Human Microvascular Endothelial Cells) (125μg/ml). After 72h of treatment RNA was isolated from AF cells for analysis of gene expression and the culture medium was collected for protein expression analysis. Results: The qRT-PCR analysis demonstrated increased gene expression of matrix metalloproteinases (MMPs) in AF cells treated with protein products of endothelial cells compared with cells from control group of AF cells: MMP-1 243.10 times (p < 0.05), MMP-2 1.37 times (p < 0.05), MMP-3 39.83 times (p < 0.05) and MMP-13 5.70 times (p < 0.05). In contrast, tissue inhibitors of metalloproteinases (TIMPs) were suppressed; TIMP-2 (0.55 time) (p < 0.05) and TIMP-3 (0.60 time) (p < 0.05) in the exposed groups. The expression of aggrecan gene (0.83 time) (p < 0.05), an important extracellular matrix component, was also reduced. MMP-1 and MMP-3 detection was performed, confirming the results of PCR by Western Blot technique. Conclusions: In this study, we observed that the proteins produced by ECs induced the MMPs expression and suppressed the TIMPs as well as the aggrecan in primary cells of the human intervertebral disc, targeting the development of potential treatments for intervertebral disc degeneration and associated discogenic pain.

Keywords: Intervertebral disc; Neovascularization pathologic; Matrix metalloproteinases.

RESUMO
Objetivo: Analisar o efeito de produtos proteicos de células endoteliais (Ces) sobre o metabolismo de células de ânulo fibroso (AF) em ambiente controlado de cultura celular in vitro. Métodos: Células de AF humano foram expandidas em camada única e tratadas com proteínas obtidas a partir do meio de cultura de células da linhaagem celular HMEC-1 (Human Microvascular Endothelial Cells) (125μg/ml). Após 72h de tratamento, isolou-se RNA das células de AF para análise da expressão gênica e coletou-se meio de cultura para análise de expressão proteica. Resultados: A análise da qRT-PCR demonstrou aumento da expressão gênica das metaloproteinases de matriz (MMPs) nas células de AF tratadas com produtos proteicos das células endoteliais, em comparação com grupo controle de células de AF: MMP-1 243,10 vezes (p < 0,05), MMP-2 1,37 vezes (p < 0,05), MMP-3 39,83 vezes (p < 0,05) e MMP13 5,70 vezes (p < 0,05). Em contraste, os inibidores teciduais das metaloproteinases (TIMPs) apresentaram supressão da expressão gênica de TIMP-2 (0,55 vezes) (p < 0,05) e TIMP-3 (0,60 vezes) (p < 0,05) nos grupos expostos. A expressão do gene agrecan (0,83 vezes) (p < 0,05), componente importante da matriz extracelular, também estava diminuída. Foi realizada detecção de MMP-1 e MMP-3, confirmando os resultados de PCR através de técnica de Western Blot. Conclusões: Neste estudo observamos que as proteínas produzidas pelas Ces induziram a expressão de MMPs e suprimiram a expressão de TIMPs e agregan nas células primárias do disco intervertebral humano, objetivando desenvolvimento de potenciais terapias no tratamento da degeneração do disco intervertebral e do discogênico associado.

Descritores: Disco intervertebral; Neovascularização patológica; Metaloproteinases da matriz.

REVIEW
Objective: To investigate the effects of the proteins products of the endothelium cells (ECs) in the fibrocellular annulus (AF) in vitro system of cell culture. Methods: Cells of AF human were amplified in monolayer cultures and treated with proteins obtained from the medium of cells HMEC-1 (human microvascular endothelial cells) (125μg/ml). After 72h of treatment, RNA was isolated from AF cells for analysis of gene expression and the culture medium was collected for protein expression analysis. Results: The qRT-PCR analysis demonstrated increased gene expression of matrix metalloproteinases (MMPs) in AF cells treated with protein products of endothelial cells compared with cells from control group of AF cells: MMP-1 243.10 times (p < 0.05), MMP-2 1.37 times (p < 0.05), MMP-3 39.83 times (p < 0.05) and MMP-13 5.70 times (p < 0.05). In contrast, tissue inhibitors of metalloproteinases (TIMPs) were suppressed; TIMP-2 (0.55 times) (p < 0.05) and TIMP-3 (0.60 times) (p < 0.05) in the exposed groups. The expression of aggrecan gene (0.83 time) (p < 0.05), an important extracellular matrix component, was also reduced. MMP-1 and MMP-3 detection was performed, confirming the results of PCR by Western Blot technique. Conclusions: In this study, we observed that the proteins produced by ECs induced the MMPs expression and suppressed the TIMPs as well as the aggrecan in primary cells of the human intervertebral disc, targeting the development of potential treatments for intervertebral disc degeneration and associated discogenic pain.

Keywords: Intervertebral disc; Neovascularization pathologic; Matrix metalloproteinases.

RESUMEN
Objetivo: Analizar el efecto de los productos de proteína de células endoteliales (Ces) en el metabolismo celular del anillo fibroso (AF) en ambiente controlado de cultura celular in vitro. Métodos: Células de AF humano se ampliaron en monolayer cultures y se trató con proteínas obtenidas a partir del medio de cultivo de células de AF: HMEC-1 (Human Microvascular Endothelial Cells) (125μg/ml). Después de 72h de tratamiento, se aisló el ARN de las células de AF para análisis de expresión genética y se recogió el medio de cultivo para análisis de expresión proteica. Resultados: El análisis de la qRT-PCR demostró una mayor expresión génica de las metaloproteínas de matriz (MMPs) en las células de AF tratadas con productos proteicos de células endoteliales, en comparación con grupo control de células de AF: MMP-1 243,10 veces (p < 0,05), MMP-2 1,37 veces (p < 0,05), MMP-3 39,83 veces (p < 0,05) y MMP13 5,70 veces (p < 0,05). En contraste, los inhibidores tisulares de las metaloproteínas (TIMPs) presentaron supresión de la expresión del gen MMP-2 (0,55 veces) (p < 0,05) y TIMP-3 (0,60 veces) (p < 0,05) en los grupos expuestos. La expresión génica de aggrecan (0,83 veces) (p < 0,05), un importante componente de la matriz extracelular, también se redujo. La detección de MMP-1 y de MMP-3 fue realizada y se confirmaron los resultados de la PCR mediante la técnica Western Blot. Conclusiones: Nuestro estudio observó que las proteínas producidas por las Ces indujeron la expresión de MMP y suprimieron la expresión del TIMP y de aggrecan en células primarias del disco intervertebral humano, con el objetivo de desarrollar posibles tratamientos para la degeneración del disco intervertebral y el dolor discogénico asociado.

Descritores: Disco intervertebral; Neovascularización patológica; Metaloproteinonas de la matriz.
INTRODUCTION

Spinal diseases related to intervertebral disc degeneration (IDD) represent a global public health problem, due to the physical and psychological limitation it causes, and the substantial associated economic losses. Around 80% of the population will experience more than one episode of back pain in the course of their lives, and more than 80% of the population exhibit some sign of disc degeneration on magnetic resonance imaging after 50 years.1-3 IDD is associated with pathological conditions of the vertebra, including spinal canal stenosis, foraminal stenosis and spinal disc herniation, often resulting in pain and/or neurological deficit and, ultimately, functional impairment.4,5

Intervertebral discs (IVDs), which are fibrocartilaginous structures located between the vertebral bodies, are responsible for the absorption, distribution and transmission of loads in the spinal column. They are macroscopically composed of annuli fibrosi (AFs), rich in type 1 collagen, and nucleus pulposus (NP), composed primarily of type 2 collagen and hydrophilic proteoglycans in a complex structure that includes cells, extracellular matrix (ECM) and 70% water.6,7

The development of IDD is related to genetic predisposition, excessive load, smoking, and the natural aging process.8,9 Many studies on the cellular mechanisms of IDD have demonstrated the importance of maintaining extracellular matrix homeostasis, striving for a balance between the synthesis and the catabolism of its components.10,11 However, natural history, as well as the biological mechanisms that lead to IDD, still have gaps to be filled.

IDD presents water loss through the NP, with a consequent reduction in disc height, and AF fissures, sometimes followed by neovascularization process and neuronal growth, as published previously by Johnson et al.12 and Stefanakis et al.13 AF invasion by vascular tissue is considered a pathological alteration, as the IVDs are avascular structures.

The cell type most commonly associated with the neovascularization process is that of the endothelium colony-forming cells, or simply endothelial cells (ECs).14,15 During the process of invasion, remodeling, and reorganization into newly formed vessels, the ECs will potentially submit the native cells of the intervertebral disc to a completely new environment.

In a study by Moon et al.,16 the cells of the already degenerated disc are seen to play an important role in the invasion of the endothelial cells, stimulating migration, the production of matrix remodeling factors and growth factors, such as the neuron growth factor.

Based on the article by Moon et al.,16 we posed the following questions: Could the opposite also be true? Could the endothelial cells be capable of modulating the gene expression of the AF cells? What is the role of endothelial cells in the disc degeneration process?

Although neovascularization has been documented in degenerated discs and in herniated disc content,17,18 there are no reports in literature that directly assess the paracrine effect of the endothelial cells on the AF cells in a controlled cell culture environment.

The goal of this study is to ascertain whether soluble endothelial cell protein products can negatively affect the metabolism of the extracellular matrix of the annulus fibrosus in a controlled in vitro cell culture environment.

METHODS

The design of this study is summarized in diagrammatic form, represented in Figure 1.

Cell Cultures

Samples of cell line HMEC-1 (Human Micro Endothelial Cells), developed and kindly provided to one of our collaborators by the United States Center for Disease Control and Prevention (CDC), were cultivated in a specific culture medium (EGM-2 MV medium; Lonza, Basel, Switzerland) until confluence of 95%, when the culture medium to be used in the treatment of the annulus fibrosus cells was collected. Phenotypic characteristics of the endothelial cells were confirmed as described previously.19

Cells of human annulus fibrosus samples were obtained from intervertebral discs removed in elective surgical procedures, and sent for cell culture under previous authorization granted by the subjects (Research Ethics Committee of the University of Pittsburgh, PA, USA, protocol number: PRO12100603). The samples were washed, fragmented and digested for 60 minutes at 37°C under gentle agitation in an F-12 culture medium containing 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 0.2% pronase (Calbiochem©, La Jolla, CA, USA) followed by 18 hours in an F-12 medium containing 5% FBS, 1% P/S and 0.02% collagenase P (Roche Diagnostics©, Indianapolis, IN, USA). The post-digestion solution was then submitted to filtration, centrifugation at 2000 rpm for 5 min, and re-suspension of the cells in F-12 medium with 10% FBS and 1% P/S, after which they were expanded in conventional culture flasks incubated at 37°C in a humidified atmosphere with 5% carbon dioxide.

All the experiments were conducted with ECs with less than 10 passes, and with AF cells, primary cells, exclusively in the first pass.

Preparation of the Culture Medium Obtained from the Endothelial Cells

ECs, in a concentration of 1x100 cells/cm², were distributed in 150 cm² culture flasks up to 95% of confluence, at which time the culture medium was replaced by Dulbecco’s Modified Eagle Medium (DMEM), with the addition of 1% FBS. This is sufficient to maintain the cell functions of the ECs, which continue for 72 hours. After this period the culture medium was collected. This solution was then submitted to successive centrifugation processes, 600g and 1500g both for 15 minutes at 4°C, to remove large cell fragments, and 100000g for 2 hours at 4°C (Beckman XL-70 ultracentrifuge, rotor SW40Ti, Beckman Coulter, Inc., Brea, CA, USA) to remove exosomes and microvesicles potentially released by the ECs.

As the end result of this process, we obtained a supernatant containing proteins secreted by the endothelial cells in the culture medium, clarified of any cell vestiges. The supernatant was concentrated through a centrifugal filter unit (Amicon Ultra-15 3 kDa NMWL; Millipore Ltd., Carrigtwohill, IRL) and submitted to protein concentration analysis using a Bicinchoninic Acid (BCA©) Protein Assay Kit (Thermo Fisher Scientific). It was then stored for up to 24 hours at 4°C, until use in the treatment of AF cells.

Figure 1. Flowchart of production of the culture medium of the ECs, isolation of the protein products, treatment regimen of the AF cells, and outcomes evaluated.
Treatment of Annulus Fibrosus Cells

As mentioned above, we used AF cells obtained from tissues resulting from surgical procedures, produced in a monolayer culture with up to 50-70% confluence, on dishes with an area of 9.5 cm² (6 wells), divided into 2 groups: A. Control (CTL): defined by AF cells in regular culture medium in the absence of treatment; B. Exposed (EXP): defined by AF cells exposed to proteins from the culture medium of the endothelial cells for 72 hours at a concentration of 125 μg/ml.

After this period, the culture medium in the treatment dishes was collected and stored at -80°C for protein detection trials using Western Blot (WB). The cells were washed twice with Hank’s balanced salt solution (HBSS) and collected for quantitative analysis of the real-time polymerase chain reaction (qRT-PCR).

Analysis of Gene Expression (qRT-PCR)

Total RNA was extracted using a RNeasy Micro Kit (Qiagen, Dusseldorf, Germany), according to the manufacturer’s instructions. The RNA concentrations were verified through spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific Inc., Wilmington, DE, USA). The real-time polymerase chain reaction (qRT-PCR) was performed using the iScript™ SYBR Green kit (Bio-Rad Laboratories, Hercules, CA, USA) in an iCycler IQ4 cycler with specific primers and probes for humans (Applied Biosystems, Foster City, CA, USA) for metalloproteinasises (MMPs), including MMP-1, MMP-2, MMP-3, MMP-13, tissue inhibitors of metalloproteinases (TIMPS), TIMP-1, TIMP-2 and TIMP-3, collagen type 1 (Col-1), aggrecan (AGC), and glyceraldehyde-3-phosphate (GAPDH) as housekeeping gene.

Table 1. Sequence of initiators (primers) used in the gene expression analysis through PCR. In the column on the left the anterograde sequences and on the right the reverse sequences.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence of Bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>GAGCCTAATCTCAGGTTGAGA / CCAAAAAGCGTGTGACGATA</td>
</tr>
<tr>
<td>MMP-2</td>
<td>GGCCCGCTGGCCCATACGCA / AGCTCTCCCTGAGGAGCCCA</td>
</tr>
<tr>
<td>MMP-3</td>
<td>CAAGGAGGCGAGCGAGCG / GCCAGCAGACAGACAGAGGG</td>
</tr>
<tr>
<td>MMP-13</td>
<td>TGCTCCTCTGACGAGCTGAC / TCTCGGAGACTGTTAAGG</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>TGGCTCTGGAGATCTGTGTTG / CGCTGTTGATAAGGTTGTCGTGTTG</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>GAATCGGTGAGGCTTCTGCTGTA / CCTGCAACAGCAGCCGGAAA</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>AGGAGCCTCTGCAACTC / GTAATGCAATGGGCCATC</td>
</tr>
<tr>
<td>Col-1</td>
<td>GCTCTCTGGACATTGGAAGA / CTTCCGTGAGCTAGAGGAGG</td>
</tr>
<tr>
<td>AGC</td>
<td>AAGATCAATCTGAGCCCTGCTGTC / TGAGACCCTGTCTGATAGGCAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCACTCCCTCAACTTTGAC / TCCACACCCCTGTTGCTGTA</td>
</tr>
</tbody>
</table>

Western Blotting Technique

Protein samples from the culture medium of the control and exposed AF cells were used in the amount of 10 μg. The samples were exposed to the SDS-Page reducer and transferred to a low fluorescence background membrane (EMD Millipore Corp., Billerica, MA, USA). The membranes were blocked by 3% milk in TBS-T (0.25% Tween-20 in TBS) for a 1-hour period, then incubated with the respective primary antibody overnight at 4°C (MMP-1, MMP-3, Abcam, Cambridge, MA, USA). The next day, the membranes were washed and incubated for 1h with the appropriate secondary antibody, diluted at a ratio of 1:25000. Using a Typhoon 9410 imager (GE Healthcare Biosciences, Piscataway, NJ, USA), the bands were visualized using green laser at 532nm in a 526-nm SP filter.

Table 2. Characteristics of the donors of intervertebral disc tissue for cell culture.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male:Female)</td>
<td>4:4</td>
</tr>
<tr>
<td>Age (Mean ± SD)</td>
<td>43.25 ± 13.22</td>
</tr>
<tr>
<td>Segment (Cervical:Lumbar)</td>
<td>7:1</td>
</tr>
<tr>
<td>Degree of Degeneration (Mean ± SD)</td>
<td>2.36 ± 0.74</td>
</tr>
<tr>
<td>Smoker (Yes:No)</td>
<td>3:5</td>
</tr>
</tbody>
</table>

Figure 2. Comparison of the AF cell cultures after treatment. On the left control group (CTL), and on the right group exposed (EXP) to the EC proteins.
by the absence of studies demonstrating the role played by the endothelial cells in this sequence of events.

Stefanakis et al\textsuperscript{13} uphold that annulus fibrosus fissures are a pathway for vascular and neuronal growth inside the AF, while Johnson et al\textsuperscript{12} demonstrated in one of their articles that proteoglycans, one of the main components of the extracellular matrix of normal discs, are potentially responsible for inhibiting vascular growth inside the disc. Ratsep et al,\textsuperscript{16} on the other hand, stressed the importance of neovascularization in the reabsorption of disc tissue of extruded hernias.

Our study is unique, as it assesses effects produced by the endothelial cells on the native AF cells, imitating the initial period of the neovascularization process. After treatment of the disc cells with EC protein products, we assessed the matrix metalloproteinase expression variations, tissue inhibitors of metalloproteinases, collagen type I and aggrecan.

Metalloproteinases are zinc-dependent endopeptidases. These enzymes are able to degrade most extracellular matrix proteins and therefore play an important role in the remodeling of the matrix. Twenty-eight MMPs have been described so far, of which 24 are detected in vertebrates and 23 in humans.\textsuperscript{25} Some are considered key enzymes in the intervertebral disc degeneration process. These include MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (Stromelysin-1) and MMP-13 (collagenase 3).\textsuperscript{26} Moon et al\textsuperscript{10} recently noted that IVd cells, in different degrees of degeneration, stimulate endothelial cells to produce key degradation markers of the extracellular matrix (MMP-2, MMP-13) and neuron growth factor, a mechanism potentially associated with discogenic pain.

In our results we were able to observe that the AF cells, when in contact with the proteins produced by the ECs, exhibited increased gene expression of key MMPs for the catabolism of MEC (MMP-1, MMP-3 and MMP-13). The two MMPs expressed the most, MMP-1 and MMP-3, also were analyzed through western blot for direct detection, and the results were confirmed by PCR.

In contrast, it was found that the gene expressions of the TIMPs, tissue inhibitors of the matrix metalloproteinases, whose main function is to neutralize the action of the MMPs, were suppressed, with TIMP-2 and TIMP-3 of 45% and 40%, respectively. We also noted suppression of the gene expression at 17%, one of the main proteins that constitute the MEC of the annulus fibrosus.\textsuperscript{24}

The imbalance between the production of MEC formers (collagen and aggrecan) and factors responsible for its degeneration (MMPs and TIMPs) allows us to predict, in vitro, that the ECs trigger a potential catabolic profile of the expression of proteins in the native AF cells during the early vascular invasion stages. However, which are the proteins known to be produced by the ECs that could potentially induce this behavior in the intervertebral disc cells?

Many factors are known to be produced by the ECs. Among these, MMP-2, MMP-13, MMP-14, Interleukin 1-beta (IL-1B) and plasminogen were detected in the culture medium of type HMEC-1 ECs.\textsuperscript{16,19} It is possible that these MMPs, IL-1B, and plasminogen, are directly related to the behavior exhibited by the AF cells. The remodeling of MEC in a dense tissue such as the AF is a prerequisite for vascular invasion as an attempt at tissue regeneration.

However, it was noted that AF healing is often inefficient, even after an intense neovascularization process.\textsuperscript{26} We raised the possibility that the growth of neovessels within the intervertebral disc degeneration process may have an effect contrary to the healing attempt, i.e., a catabolic effect, thus increasing the breakage of the MEC and suppressing the production of factors responsible for its synthesis and consequent homeostasis.

CONCLUSION

Although the study submitted has the limitations inherent to in vitro studies, we were able to observe, in a controlled environment, the potential deleterious effects of the endothelial cells on the primary cells of the human intervertebral disc, and thereby design a microbiological scenario possibly established during the intervertebral disc neovascularization and degeneration process.
Additional in vitro studies, besides confirming the results in vivo, are necessary to obtain additional information about the factors produced and secreted by the endothelial cells during the vascular and neuronal invasion process, with the aim of developing potential therapies for the treatment of intervertebral disc degeneration and associated discogenic pain.

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All authors declare no potential conflict of interest concerning this article.

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