Published in final edited form as:

J Orthop Res. 2016 August ; 34(8): 1466–1474. doi:10.1002/jor.23298.

Catabolic Effects of Endothelial Cell-Derived Microparticles on Disc Cells: Implications in Intervertebral Disc Neovascularization and Degeneration

Pedro H. I. Pohl^{1,2}, Thomas P. Lozito³, Thais Cuperman³, Takashi Yurube^{1,4}, Hong J. Moon^{1,5}, Kevin Ngo¹, Rocky S. Tuan³, Claudette St. Croix⁶, Gwendolyn A. Sowa^{1,7}, Luciano M. R. Rodrigues², James D. Kang¹, and Nam V. Vo¹

¹Ferguson Laboratory for Orthopaedic and Spine Research, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh – USA

²Spine Surgery Group, Discipline of Orthopaedic Surgery and Traumatology, ABC Medical School (FMABC), Sao Paulo – Brazil

³Center for Cellular and Molecular Engineering, University of Pittsburgh School of Medicine, Pittsburgh – USA

⁴Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, Kobe – Japan

⁵Department of Neurosurgery, College of Medicine, Korea University, Seoul – Republic of Korea

⁶Center for Biologic Imaging, Environmental and Occupational Health, University of Pittsburgh, Pittsburgh - USA

⁷Department of Physical Medicine and Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh – USA

Abstract

Neovascularization of intervertebral discs, a phenomenon considered pathological since normal discs are primarily avascular structures, occurs most frequently in annulus fibrosus (AF) of degenerated discs. Endothelial cells (ECs) are involved in this process, but the mechanism of the interaction between AF and endothelial cells is unclear. In this study we evaluated the effects on matrix catabolic activity of AF cells by the extracellular endothelial microparticles (EMPs) and soluble protein factors (SUP fraction) produced from ECs. Passage 1 human AF cells grown in monolayer cultures were treated for 72 hours with 250µg of EMPs or SUP fraction isolated from culture of the microvascular endothelial cell line, HEMC-I. Live-cell imaging revealed uptake of EMPs by AF cells. RT-PCR analysis demonstrated increased mRNA expression of MMP-1 (50.3

Corresponding Author: Nam Vo, Ph.D., Department of Orthopaedic Surgery, Biomedical Science Tower EBST1643, University of Pittsburgh School of Medicine, Pittsburgh (PA) 15261, USA; Phone: +1 (412) 648-1092 / Fax: +1 (412) 383-5307. von@upmc.edu.

Author Contributions: Vo NV, Kang JD, Sowa GA, Tuan RS, Rodrigues LMR, Pohl PHI (project development, experimental design, data interpretation, intellectual inputs), Pohl PHI, Yurube T, Moon HJ, Ngo K, Croix C (disc cells collection and culture, live cell imaging, PCR and western blot), Lozito TP, Cuperman T, Pohl PHI (endothelial cells culture, western blot, MMP activity assay). All authors were actively involved in draft, prepare and review this manuscript.

Conflict of Interest: There is no conflict of interest related to this study.

fold), MMP-3 (4.5 fold) and MMP-13 (5.5 fold) in AF cell cultures treated with EMPs compared to untreated control. Western analysis also demonstrated increased MMP protein expression in EMP-treated AF cells. AF cells treated with the SUP fraction also exhibited a dramatic increase in MMP mRNA and protein expression. Increased MMP expression is primarily due to EMP or SUP stimulation of AF cells since EMPs or SUP fraction alone contained negligible amount of MMPs. Interestingly, MMP activity was elevated in AF cell cultures treated with EMPs but not with SUP. This study revealed enhanced matrix catabolism as a molecular consequence of action of ECs on AF cells via EMPs, which might be expected during neo-angiogenesis of degenerating disc.

Keywords

Intervertebral disc degeneration; Neo-angiogenesis; Endothelial cells; Microparticles; Matrix metalloproteinases

Introduction

Spine pathologies related to intervertebral disc degeneration (IDD) represent a worldwide public health problem due to the associated physical and psychosocial disability as well as substantial economic losses. About 80% of the world's population will experience one or more episodes of back pain during their lifetime with 39% presenting with IDD^{1; 2}. IDD has been linked to several spine-related conditions including spinal stenosis, disc herniation and radiculopathy resulting in pain and/or neurological deficits^{3; 4}. The development of IDD is associated with genetic predisposition, mechanical overload, aging, and other extrinsic factors such as tobacco smoking^{5; 6}. While it is well documented that disruption of disc matrix homeostasis leads to IDD, the molecular mechanisms of how the balance between disc matrix synthesis and breakdown become perturbed remain poorly understood^{7; 8}.

IDD is closely correlated with AF fissures, neo-innervation, and neovascularization^{9; 10}. Multiple inductive factors are involved in vascular and nerve ingrowth, including the vascular endothelium growth factor (VEGF) and nerve growth factor (NGF), both of which have been reported to be present in degenerative discs¹¹⁻¹⁴. Vascular ingrowth is considered pathological, since normal discs are primarily avascular structures. David and coworkers also observed that disc expression of neovascularization growth factors is associated with post-surgical pain, implicating this process in a clinically relevant pathology¹⁵. Moreover, neovascularization of degenerated discs occur most frequently in the annulus fibrosus (AF) tissue¹⁶, underscoring the importance of studying the interaction between the resident AF cells and the invasive vasculature cells.

It is well established that cells can communicate using several different mechanisms, including direct cell-cell contact and cell fusion, or indirectly through paracrine signaling by soluble factors such as cytokines, growth factors, and prostaglandins¹⁷⁻¹⁹. Recently a new mechanism of cellular communication was identified in which cells can influence their neighboring cells through the secretion of microparticles (MPs). MPs, also referred to as exosomes or microvesicles, are secreted membrane-enclosed vesicles. Collectively called extracellular vesicles, these vesicles harbor complex RNA and protein mixture which set them apart from the free RNAs and proteins, i.e., not encased in membrane vesicles, in the

extracellular milieu. MP-mediated cell-cell communication currently represents one of the most rapidly growing fields in biology and medicine research²⁰⁻²². For instance, a current active area of research focuses on elucidating how MP proteins promote cancer progression and metastasis²³.

MPs consist of particles smaller than 1 μm in diameter, which contain subcellular components, e.g., membrane, cytoplasm, proteins, mRNA and microRNA, originating from their parental cells. MPs can signal and alter the phenotypic program of adjacent or distant cells. MPs can be released by multiple cell types in the human body, but endothelial cells (ECs), the primary cells of the vasculature, are also known to produce MPs²⁴⁻²⁶. Recently we demonstrated that human AF cells derived from degenerated discs stimulate endothelial cells to produce key factors involved in matrix breakdown, angiogenesis and innervation^{11; 27}, underscoring important interactions between endothelial cells and disc cells. Therefore we postulated that during the neovascularization process, invasion of endothelial cells and their interaction with the resident AF cells might promote a catabolic microenvironment in AF tissue¹¹, and MPs have the potential be involved in this interaction.

Endothelial microparticles (EMPs) have been studied widely²⁸⁻³¹, in part because of the ease of extraction and purification from peripheral blood and EC cultures. Several studies linked EMPs to extracellular matrix metabolism regulation and to different diseases such as acute coronary syndrome, severe hypertension, metabolic syndrome, renal disease, diabetes and oncologic processes^{21; 22; 32-36}. Neovascularization in degenerated, herniated, and injured discs^{16; 27} inevitably results in invasion of disc tissue by vascular cells. However, there are no literature reports that evaluate the effects of EMPs on the resident IVD cell metabolism. The inherent role of ECs in vascular invasion¹¹ raises an important question of whether EC products such as EMPs promote disc matrix imbalance and contribute to IDD. The objective of the present study is to determine the effects of EMPs on matrix catabolic activity of AF cells.

Methods

AF Cell Culture

Human AF was obtained from elective cervical spine surgical procedures (n = 14; male:female = 10:4; mean age $\pm$ SE = 47.4 $\pm$ 2.5; mean Pfirrmann grade $\pm$ SE = 2.4 $\pm$ 0.1³⁷). Different surgical specimens were expanded and cultured separately, for all the assays. The Institutional Review Board for human subjects approved the experimental protocol number PRO12100603 at the University of Pittsburgh. Each sample of AF tissue were washed, minced and digested for 60 minutes at 37°C with gentle agitation in sterile F-12 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 F-12 medium containing 5% fetal bovine serum (FBS), 1% P/S, and 0.02% collagenase P (*Roche Diagnostics*®, Indianapolis, IN, USA). The digested solution was filtered through 70- μm -pore size nylon filter (*Millex*®, Tullagreen, Carrigtwohill, IRL) and centrifuged at 2000 rpm for 5 minutes. The cell pellet was re-suspended and plated in F-12 medium with 10% FBS and 1% P/S, and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide.

AF cells were cultured in F-12 medium containing 10% FBS until passage 1 when the medium were changed to F-12 serum-free containing 1% insulin, transferrin and sulfate (ITS) solution (*Invitrogen*, Carlsbad, CA, USA), or serum-free phenol-free (SFPPF) medium also containing 1% ITS, depending on the assays to be performed as described below.

Endothelial Cell Culture and Microparticle Isolation

Human microvascular endothelial cell line (HMEC-I) ³⁸ was cultured in EC medium (EGM-2 MV medium; *Lonza*, Basel, Switzerland) in monolayer. Phenotypic features of this EC line were confirmed as previously described ²¹. All experiments were performed using HMEC-I culture in EGM-2 MN medium supplemented with 10% FBS or 1% FBS.

ECs were seeded at 100 cells/cm² in 150 cm² dishes and cultivated in 10% FBS EC medium. After the cells reached 95% confluence, the medium was replaced with Dulbecco's Modified Eagle Medium (DMEM) and incubated for three days. EC culture conditioned media was then collected and centrifuged once at 600g followed by 1500g, each time for 15 min at 4°C, to remove cellular debris. The clarified medium was subjected to ultracentrifugation at 100,000g for 2 hours at 4°C in a Beckman XL-70 Ultracentrifuge (SW40Ti rotor, *Beckman Coulter Inc.*, Brea, CA, USA) to partition into the endothelial microparticle-containing pellet fraction (EMPs) and the supernatant (SUP) fluid fraction cleared of microparticles.

The EMP pellet fraction was suspended in serum free DMEM. The SUP fraction was concentrated about 5 fold using the spin columns (Amicon Ultra-15 3 kDa NMWL; *Millipore Ltd.*, Carrigtwohill, IRL). EMP and SUP protein concentrations were quantified using Bicinchoninic (BCA^{TM}) Protein Assay Kit (*Thermo Fisher Scientific*). Fresh EMP and SUP samples were used to treat AF cells (Fig. 1A).

Electron Microscopy: The presence of EMPs was confirmed by electron microscopic protocol adapted from Dolo et al ³⁹. Briefly, ultracentrifugation EMP-containing pellets were resuspended in PBS and applied to collodion-coated grids, negatively stained with 1% phosphotungstic acid, pH 7.0, and observed with transmission electron microscopy (JEM-1011 Electron Microscope) (Fig. 1B).

Treatments of AF cells with EMP and SUP fractions

Cultures of passage 1 human AF cells in 6 well plates at 50-70% confluence were treated under three conditions: EMPs (250 µg of protein/well), SUP (250 µg of protein/well), and regular medium as control for a period of 72 hrs. After treatment, culture conditioned media were collected and stored at -80°C. The cells were washed 2 times with HBSS followed by cell lysate collection and storage at -80°C. For qRT-PCR, F-12 medium with 10% FBS and 1% P/S was used. For Western analysis and MMP activity assay, Serum free F-12 medium supplemented with 1% ITS and 1% P/S was used in order to avoid serum interference on MMP activity assay.

Assaying Uptake of EMPs by AF Cells

DiO-Labeled EMPs Production—After trypsinization, the AF cell pellet was collected by centrifugation (5 min at 600 g) and re-suspended at 10^6 cells/ml in fresh EC medium with no FBS. Vybrant® DiO (*Invitrogen*), a membrane lipophilic tracker, was added (5 µg/ml of cell suspension) and incubated for 20 min at 37°C to label the cells. After labeling, cells were washed three times with serum free EC medium and cultured for subsequent isolation of DiO-labeled EMPs (Fig. 2A).

Live Cell Imaging—P1 AF cells (30×10^3 cell/well suspended in 1 ml of fresh media) were plated and cultivated in 35 mm glass surfaced petri dishes (*MatTek Corporation*) until 50% confluence. The AF cells were stained with Cell Tracker™ Red CMTPX and DAPI nuclear stain (*Invitrogen*). The DiO labeled EMPs were added to the petri dish (250 µg of protein/well) containing the AF cells and incubated for four hours. Time-lapse confocal microscopy (*Nikon Eclipse Ti* – Nikon's sweptfield confocal system) was performed in 10 different fields that were imaged every 20 minutes for a total of 240 minutes (Fig. 2B).

Gene Expression Analysis by Real-Time PCR

Total RNA was extracted from AF cells in each condition using RNeasy Micro Kit (Qiagen) according to the manufacturer's instruction. RNA concentration was measured by spectrophotometry (Nanodrop ND-1000, *Thermo Fisher Scientific Inc.*, Wilmington, DE). Real time reverse transcription-polymerase chain reactions (RT-PCR) were performed with validated primers (Table 1) using the reagents and protocol per the Bio-Rad iScript (iCycler IQ4, *Bio-Rad laboratories*, Hercules, CA, USA). Data were normalized to GAPDH and relative mRNA expression was calculated using the $\Delta\Delta C_t$ method comparing gene expression of AF cells from AF-EMPs treated, AF-SUP treated and AF-media control conditions⁴⁰.

Western Blotting

Proteins (10µg) from culture-conditioned medium of treated AF cells were resolved on reducing SDS-PAGE (4-12% gradient gel) and transferred to a low fluorescence background pure nitrocellulose membrane (*EMD Millipore Corp.*, Billerica, MA, USA). Membranes were blocked in 5% milk in TBS-T (0.25% Tween-20 in tris buffered saline (TBS)) for 1 h and incubated overnight at 4°C with respective antibodies against MMP-1, MMP-2, MMP-3, TIMP-1 or TIMP-2 (*Abcam*, Cambridge, MA, USA) in 1% milk/TBS-T. The membranes were washed and incubated for 1 h with the appropriate Alexa Fluor 488-conjugated secondary antibody (1:2000 dilution) in 1% milk/TBS-T. The membranes were washed 3× with TBS-T and imaged using the ChemiDoc Imaging System (*Bio-Rad*, Hercules, CA, USA) using a 532 nm excitation laser and a 526 nm SP filter.

MMP Activity Assay

Culture conditioned media from AF cells treated with EMPs or SUP as well as media fractions from EMPs alone resuspended in naïve medium (not culture with AF cells) and SUP alone diluted in naïve medium (not cultured with AF cells) were collected and snap

frozen at -80°C for MMP enzymatic activity assessment. Conditioned media from untreated AF cells and naïve media alone were also used as controls.

Protein samples (10 μg) were incubated with 5 nM MMP Fluorogenic Substrate XI (5-FAM-P-Cha-G-Nva-HA-Dap(QXL^{TM} 520)-NH₂) (*Anaspec*, Fremont, CA, USA) for 60 min, and fluorescence was measured on a BioTek microplate reader (Ex/Em=494/521 nm). This fluorogenic substrate can be recognized and cleaved by MMP-1, 2, 8, 12, 13. MMP activities of AF-conditioned media samples were calculated by subtracting fluorescence measurements of corresponding controls for each sample.

Statistics

All independent samples, i.e., AF cells derived from different donors, had the results obtained in duplicates, with $n=3-7$ as indicated for each outcome measure. Mann-Whitney U test was performed to compare the conditions and significant differences were defined at $p < 0.05$. IBM SPSS statistics version 21 was used for statistical analysis.

Results

Uptake of EMPs by AF cells

Time-lapse confocal microscopy revealed uptake of green DiO-labeled EMPs by AF cells. The uptake of EMP (yellow arrows) was confirmed by obtaining images in two planar orientations (fig. 2A and B and video 1). In this field sample (Fig. 2B), an EMP was observed to make contact with the AF cell surface within 20 minutes and get internalized within 40-60 minutes after EMP addition to AF cell culture, demonstrating the uptake of EMPs by AF cells. Aside from revealing cell plasma membrane and nucleus, our live cell imaging technique does not allow for determination of specific intracellular localization of EMP. However the signal from the green DiO-labeled EMP was greatly diminished at 60 minutes near the cell surface, suggesting breakdown of EMP upon internalization.

EMPs upregulate MMP mRNA expression in AF cells

RT-PCR analysis showed increased mRNA expression of MMPs in AF cells treated with EMPs. Compared to untreated AF cells, mRNA expression of EMP-treated AF cells was increased for MMP-1 (50.3 ± 26.6 fold) ($p=0.008$), MMP-3 (4.5 ± 3.5 fold) ($p=0.026$) and MMP-13 (5.5 ± 5.2 fold) ($P=0.001$). Similarly compared to untreated AF cells, mRNA expression of SUP-treated AF cells was also increased for MMP-1 (161.8 ± 77.1 fold) ($p=0.008$), MMP-2 (1.4 ± 0.3 fold) ($p=0.001$), MMP-3 (54.1 ± 48.9 fold) ($p=0.001$) and MMP-13 (5.0 ± 1.3 fold) ($p=0.001$) (Fig. 3A). With the exception of MMP-13, SUP treatment induced greater mRNA expressions of MMP-1, MMP-2, and MMP-3 in AF cells than those seen in EMP treatment.

In contrast, mRNA expression of tissue inhibitors of metalloproteinases (TIMPs) in EMP-treated AF cells decreased or remained unchanged compared to untreated control. TIMP-2 mRNA was down regulated in AF cells exposed to EMPs (0.63 ± 0.18 fold) ($p=0.029$) or SUP (0.55 ± 0.19 fold) ($p=0.029$). In addition, TIMP-3 mRNA expression was significantly suppressed when exposed to SUP (0.60 ± 0.30 fold) ($p=0.029$) in comparison to control AF

cells (Fig. 3B). TIMP-1 mRNA level in AF cells did not change following EMP or SUP treatment.

The mRNA level of aggrecan in AF cells decreased after exposure to EMPs (0.79 ± 0.12 fold) ($p=0.001$) or SUP (0.86 ± 0.15 fold) ($p=0.026$). Collagen type 1 did not show statistical difference in mRNA expression in AF cells exposed to EMPs or SUP compared to untreated control (Fig. 3C).

EMPs upregulate MMP protein expression in AF cells

Western analysis was performed to determine MMP and TIMP protein expression in the culture conditioned media of AF cells treated with EMPs or SUP. Low levels of MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2, were detected in conditioned media of untreated AF cells, but expression of these proteins increased substantially in the culture conditioned media of AF cells following 72h exposure to EMPs or SUP (Fig. 4A). A total of four independent experiments including quantification were performed (Fig. 4B). Western analysis revealed the majority of the MMPs and TIMPs to have come from EMP- or SUP-treated AF cells, not EMP or SUP fraction alone (Supplemental Fig.1). In addition, SUP-treated AF cells secreted mostly inactive MMPs (e.g., MMP-3 inactive pro-enzyme is present and active form is absent) while EMP-treated AF cells produced both inactive and active MMP-3 forms; EMPs alone contain mostly active MMP-3 form (Supplemental Fig.1).

Treatment with EMPs or SUP fractions increased both MMP protein expression in AF cells, consistent with mRNA results. AF cell TIMP-1 and TIMP-2 protein expression were stimulated while mRNA expression of the latter decreased and the former remained unchanged after exposure to EMPs and SUP. In the absence of AF cells, TIMP-1 and TIMP-2 protein levels were not detectable in EMPs or SUP alone (data not shown), suggesting that these TIMP proteins were produced by AF cells following stimulation by EMPs or SUP. Similarly, the levels of MMPs in EMPs or SUP alone were at least 10× lower than those in the conditioned media of AF cells treated with EMPs or SUP (data not shown), indicating that the majority of the MMPs were produced by AF cells following their treatment with EMPs or SUP.

AF cell MMP activity in the presence of EMPs and SUP

Cell-free controls contained little or no MMP activity in the naïve media control and the SUP fraction. Likewise, conditioned media from untreated AF cells and SUP-treated AF cells showed little or no MMP activity. In contrast, conditioned media of EMP-treated AF cells exhibited a large increase of MMP activity ($p=0.001$) (Fig. 5) compared to that from untreated AF cells, and most of this MMP activity is present in the EMPs since there was a substantial amount of MMP enzymatic activity in the EMP fraction alone.

Discussion

New vascular ingrowth is well documented in injured and degenerated discs, predominantly in the AF region^{11-15; 27}. Annulus fissures are reported to be conducive to ingrowth of blood vessels and nerves in the outer layer of degenerated discs¹⁰. Ratsep et al. also demonstrated neovascularization in reabsorption of herniated discs, suggesting the importance of

neovascularization in matrix remodeling and metabolism. Neovascularization creates an altered disc microenvironment in which some resident cells are situated in close proximity to endothelial cells⁴¹, the principal cellular constituent of blood vessels. This raises the question of whether cellular communication occurs between ECs and AF cells and how such communication ultimately influences disc matrix homeostasis. As a first step to elucidate this potentially important cell-cell communication, our goal in this study was to determine the effects of the microparticles and other secreted factors produced by ECs on disc AF cell metabolism using a cell culture model system.

Human AF cell cultures exposed to microparticles and soluble proteins secreted from endothelial cells drastically up regulated mRNA expression of the major MMPs (MMP-1, MMP-3 and MMP-13) and down regulated mRNA expression of aggrecan. These results suggested that endothelial cells produce factors, both in the microparticles and soluble protein fractions, which induce catabolic response in AF cells. A possible mechanism of action on AF cells by microparticles is through their internalization by the AF cells, as demonstrated by our live-cell imaging experiment. Interestingly, both the microparticles and the soluble protein fractions induced production of MMP-1 protein in AF cells as determined by western blot analysis.

No MMP activity was detected in untreated AF cells while some MMP activity was detected in EMP fraction alone. However, MMP activity was modestly enhanced in condition media of AF cells exposed to endothelial microparticles, indicating that EMPs play a role in MMPs activation during this process. The absence of MMP activities in soluble protein fraction (SUP) treated AF cell culture media suggested that the MMP proteins induced by the SUP fraction, as detected by western blotting, were mostly inactive (Supplemental Fig.1).

Perturbation of disc cell matrix homeostatic balance by endothelial cell microparticles

Normal healthy intervertebral discs tightly regulate a delicate balance between production and degradation of ECM components to maintain matrix homeostasis^{8; 42}. In fact, the presence of intact aggrecan has been shown to block neo-innervation and neovascularization in disc tissue⁹. Thus invasion of ECs requires aggrecan abundance to be reduced at the site of neovascularization⁹, either by reducing new aggrecan expression or breaking down the existing aggrecan structure. Consistent with this idea is our finding of lowered expression of aggrecan and increased expression of key IDD-associated MMPs in AF cells following exposure to EMPs as well as the soluble protein fraction. Our findings suggest that ECs produce factors, both in the form of microparticles and soluble proteins, which can induce perturbation of matrix homeostasis of IVD by upregulating the anabolic factors, i.e., MMPs, and repressing the expression of matrix aggrecan gene expression.

Microparticles and soluble proteins of endothelial cells differentially modulate the MMPs commonly associated with IDD, including MMP-1, MMP-2, MMP-3 and MMP-13⁴². While treatment of AF cells with either EMPs or SUP protein fraction upregulated expression of these MMPs, SUP treatment produced much greater induction, particularly of MMP-1 and MMP-3 expression, than that seen in EMP treatment of AF cells. Such differential regulation of MMP expression implies that the EMPs and SUP fractions harbor

dissimilar inducing factors or relative amount of these factors, the identification and confirmation of which will require further studies.

The increase of MMP-1 and MMP-3 mRNAs corresponds with their increased protein expression as well as the increased MMP activity. However, the increase in TIMP-1 and TIMP-2 protein expression did not reflect their mRNA expression levels. The disconnection between TIMP mRNA and protein expression indicates that transcriptional regulation of TIMP-1 is distinct from its translational regulation. In other words, SUP and EMPs appear to stimulate TIMP-1 protein production post-transcription, i.e., upregulating translation and possibly post-translation steps.

Uptake of EMPs by annulus fibrosus cells

EMPs can affect AF cell metabolism through several conceivable mechanisms: by binding to AF cell surface and emptying their cargo into the cells, binding to cell surface membrane and triggering signaling pathways, or entering the cells and releasing cargo intracellularly. The results from our live cell imaging experiment demonstrated uptake of EMPs by AF cells. Although the techniques used limited the overall assessment of absolute percentage of AF cells demonstrating EMP uptake, the biologic responses observed suggest that this event occurred at an appreciable rate. However, this does not exclude the possibility that EMPs could act on AF cells through other mechanisms. Nevertheless our finding demonstrate that a possible mode of action is through internalization of EMPs by AF cells.

It has been described that microparticles enter into cells by endocytosis and membrane fusion ⁴³. Jansen and coworkers ²⁰ reported cellular uptake of EMPs is mediated by Annexin I/Phosphatidylserine/coronary receptor in coronary endothelial cells. Whether AF cells internalize EMPs through this receptor-mediated endocytosis remained to be investigated.

Limitations and concluding remarks

Our study has several limitations. We used AF cells isolated exclusively from surgical degenerative disc specimen since neovascularization occurs mostly in degenerative disc tissue. Due to lack of availability of nondegenerative disc tissue, we did not study AF cells isolated from normal disc controls. Second, the current study focused exclusively on the effects of secreted microparticles and soluble factors in the culture media of endothelial cells on AF cells using a 2-D cell culture model system. This represents just one of several components in the multi-step process of neovascularization which occurs within the 3-D loaded extracellular matrix in vivo.

Although ECs are known to manufacture a considerable amount of MMPs, MT-MMPs and urokinase plasminogen activator (UPA) ⁴⁴, the complete molecular content and composition of EMPs and their cargo has not been determined. Determination of mRNA, protein and miRNA content of EMPs will be needed in order to identify the factors responsible for modulating the AF cell metabolism observed in this study.

The current study complemented our previously reported work demonstrating that AF cells from degenerated discs stimulate endothelial cell production of factors known to induce matrix degradation, angiogenesis, and innervation ¹¹. The current study also revealed EC

microparticles as a potentially novel mediator of cell-cell communication in disc neoangiogenesis. Our studies provide an important groundwork for future research to identify the role of EC microparticles and soluble factors as novel mediators responsible for promoting catabolic response in AF cells and key signaling pathways involved in the extracellular matrix degradation during neo-vascularization and neo-innervation in intervertebral disc degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge professor Daniel C. Devor (Department of Cell Biology and Physiology, University of Pittsburgh) who kindly provided HMEC-I. We also thank Dr. Bing Wang (UPMC Molecular Therapeutics Lab) and Gregory A. Gibson (Center for Biological Imaging) for their technical support in EMP isolation and live cell imaging, respectively. We thank the members of Ferguson Laboratory and Drs. Caio P. Barbosa and Bianca Bianco of The Post Graduation Program of ABC Medical School for continuing support and friendship.

Role of the Funding Source: This study was supported in part by The ISSLS Macnab/Larocca Research Fellowship Award (Pedro Pohl), NIH grant R01 AG044376-01 (Nam Vo), and The Albert B. Ferguson Fund of The Pittsburgh Foundation. Pedro Pohl also received funding support from the Program Science Without Borders sponsored by CAPES Foundation (Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior), Ministry of Education of Brazil.

References

- Schwarzer AC, Aprill CN, Derby R, et al. The prevalence and clinical features of internal disc disruption in patients with chronic low back pain. *Spine (Phila Pa 1976)*. 1995; 20:1878–1883. [PubMed: 8560335]
- Peng B, Fu X, Pang X, et al. Prospective clinical study on natural history of discogenic low back pain at 4 years of follow-up. *Pain physician*. 2012; 15:525–532. [PubMed: 23159971]
- Bendix T, Kjaer P, Korsholm L. Burned-out discs stop hurting: fact or fiction? *Spine (Phila Pa 1976)*. 2008; 33:E962–967. [PubMed: 19050574]
- Rodrigues LM, Theodoro TR, Matos LL, et al. Heparanase isoform expression and extracellular matrix remodeling in intervertebral disc degenerative disease. *Clinics (Sao Paulo)*. 2011; 66:903–909. [PubMed: 21789398]
- Battie MC, Videman T, Gibbons LE, et al. 1995 Volvo Award in clinical sciences. Determinants of lumbar disc degeneration. A study relating lifetime exposures and magnetic resonance imaging findings in identical twins. *Spine (Phila Pa 1976)*. 1995; 20:2601–2612. [PubMed: 8747238]
- Vo N, Seo HY, Robinson A, et al. Accelerated aging of intervertebral discs in a mouse model of progeria. *J Orthop Res*. 2010; 28:1600–1607. [PubMed: 20973062]
- Studer RK, Aboka AM, Gilbertson LG, et al. p38 MAPK inhibition in nucleus pulposus cells: a potential target for treating intervertebral disc degeneration. *Spine (Phila Pa 1976)*. 2007; 32:2827–2833. [PubMed: 18246004]
- Wang D, Nasto LA, Roughley P, et al. Spine degeneration in a murine model of chronic human tobacco smokers. *Osteoarthritis Cartilage*. 2012; 20:896–905. [PubMed: 22531458]
- Johnson WE, Caterson B, Eisenstein SM, et al. Human intervertebral disc aggrecan inhibits endothelial cell adhesion and cell migration in vitro. *Spine (Phila Pa 1976)*. 2005; 30:1139–1147. [PubMed: 15897827]
- Stefanakis M, Al-Abbasi M, Harding I, et al. Annulus fissures are mechanically and chemically conducive to the ingrowth of nerves and blood vessels. *Spine (Phila Pa 1976)*. 2012; 37:1883–1891. [PubMed: 22706090]

11. Moon HJ, Yurube T, Lozito TP, et al. Effects of secreted factors in culture medium of annulus fibrosus cells on microvascular endothelial cells: elucidating the possible pathomechanisms of matrix degradation and nerve in-growth in disc degeneration. *Osteoarthritis Cartilage*. 2013
12. Binch AL, Cole AA, Breakwell LM, et al. Expression and regulation of neurotrophic and angiogenic factors during human intervertebral disc degeneration. *Arthritis research & therapy*. 2014; 16:416. [PubMed: 25209447]
13. Kokubo Y, Uchida K, Kobayashi S, et al. Herniated and spondylotic intervertebral discs of the human cervical spine: histological and immunohistological findings in 500 en bloc surgical samples. Laboratory investigation. *J Neurosurg Spine*. 2008; 9:285–295. [PubMed: 18928227]
14. Purmessur D, Freemont AJ, Hoyland JA. Expression and regulation of neurotrophins in the nondegenerate and degenerate human intervertebral disc. *Arthritis research & therapy*. 2008; 10:R99. [PubMed: 18727839]
15. David G, Ciurea AV, Iencean SM, et al. Angiogenesis in the degeneration of the lumbar intervertebral disc. *Journal of medicine and life*. 2010; 3:154–161. [PubMed: 20968201]
16. Lama P, Le Maitre CL, Dolan P, et al. Do intervertebral discs degenerate before they herniate, or after? *The bone & joint journal*. 2013; 95-B:1127–1133. [PubMed: 23908431]
17. Vadala G, Studer RK, Sowa G, et al. Coculture of bone marrow mesenchymal stem cells and nucleus pulposus cells modulate gene expression profile without cell fusion. *Spine (Phila Pa 1976)*. 2008; 33:870–876. [PubMed: 18404106]
18. Gilbertson L, Ahn SH, Teng PN, et al. The effects of recombinant human bone morphogenetic protein-2, recombinant human bone morphogenetic protein-12, and adenoviral bone morphogenetic protein-12 on matrix synthesis in human annulus fibrosis and nucleus pulposus cells. *Spine J*. 2008; 8:449–456. [PubMed: 18455111]
19. Le Maitre CL, Freemont AJ, Hoyland JA. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis research & therapy*. 2005; 7:R732–745. [PubMed: 15987475]
20. Jansen F, Yang X, Hoyer FF, et al. Endothelial microparticle uptake in target cells is annexin I/ phosphatidylserine receptor dependent and prevents apoptosis. *Arterioscler Thromb Vasc Biol*. 2012; 32:1925–1935. [PubMed: 22701020]
21. Lozito TP, Tuan RS. Endothelial cell microparticles act as centers of matrix metalloproteinase-2 (MMP-2) activation and vascular matrix remodeling. *J Cell Physiol*. 2012; 227:534–549. [PubMed: 21437907]
22. Tramontano AF, Lyubarova R, Tsiakos J, et al. Circulating endothelial microparticles in diabetes mellitus. *Mediators Inflamm*. 2010; 2010:250476. [PubMed: 20634911]
23. Tkach M, Thery C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell*. 2016; 164:1226–1232. [PubMed: 26967288]
24. de Jong OG, Verhaar MC, Chen Y, et al. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *Journal of extracellular vesicles*. 2012:1.
25. Bakouboula B, Morel O, Faller AL, et al. Significance of membrane microparticles in solid graft and cellular transplantation. *Front Biosci*. 2011; 16:2499–2514.
26. Hugel B, Martinez MC, Kunzelmann C, et al. Membrane microparticles: two sides of the coin. *Physiology (Bethesda)*. 2005; 20:22–27. [PubMed: 15653836]
27. Ratsep T, Minajeva A, Asser T. Relationship between neovascularization and degenerative changes in herniated lumbar intervertebral discs. *Eur Spine J*. 2013
28. Bernal-Mizrachi L, Jy W, Fierro C, et al. Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes. *Int J Cardiol*. 2004; 97:439–446. [PubMed: 15561331]
29. Chironi GN, Boulanger CM, Simon A, et al. Endothelial microparticles in diseases. *Cell Tissue Res*. 2009; 335:143–151. [PubMed: 18989704]
30. Horstman LL, Jy W, Jimenez JJ, et al. New horizons in the analysis of circulating cell-derived microparticles. *Keio J Med*. 2004; 53:210–230. [PubMed: 15647627]
31. Jimenez JJ, Jy W, Mauro LM, et al. Elevated endothelial microparticles in thrombotic thrombocytopenic purpura: findings from brain and renal microvascular cell culture and patients with active disease. *Br J Haematol*. 2001; 112:81–90. [PubMed: 11167788]

32. Habersberger J, Strang F, Scheichl A, et al. Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. *Cardiovasc Res.* 2012
33. Porto I, Biasucci LM, De Maria GL, et al. Intracoronary microparticles and microvascular obstruction in patients with ST elevation myocardial infarction undergoing primary percutaneous intervention. *Eur Heart J.* 2012
34. Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, et al. Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *Am J Pathol.* 2008; 173:1210–1219. [PubMed: 18772329]
35. Preston RA, Jy W, Jimenez JJ, et al. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension.* 2003; 41:211–217. [PubMed: 12574084]
36. Faure V, Dou L, Sabatier F, et al. Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost.* 2006; 4:566–573. [PubMed: 16405517]
37. Pfirrmann CW, Metzdorf A, Zanetti M, et al. Magnetic resonance classification of lumbar intervertebral disc degeneration. *Spine (Phila Pa 1976).* 2001; 26:1873–1878. [PubMed: 11568697]
38. Ades EW, Candal FJ, Swerlick RA, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *The Journal of investigative dermatology.* 1992; 99:683–690. [PubMed: 1361507]
39. Dolo V, Ginestra A, Ghersi G, et al. Human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in gelatinolytic activities. *Journal of submicroscopic cytology and pathology.* 1994; 26:173–180. [PubMed: 8019942]
40. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
41. Adams MA, Stefanakis M, Dolan P. Healing of a painful intervertebral disc should not be confused with reversing disc degeneration: implications for physical therapies for discogenic back pain. *Clin Biomech (Bristol, Avon).* 2010; 25:961–971.
42. Vo NV, Hartman RA, Yurube T, et al. Expression and regulation of metalloproteinases and their inhibitors in intervertebral disc aging and degeneration. *Spine J.* 2013; 13:331–341. [PubMed: 23369495]
43. Svensson KJ, Christianson HC, Wittrup A, et al. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem.* 2013; 288:17713–17724. [PubMed: 23653359]
44. Lacroix R, Sabatier F, Mialhe A, et al. Activation of plasminogen into plasmin at the surface of endothelial microparticles: a mechanism that modulates angiogenic properties of endothelial progenitor cells in vitro. *Blood.* 2007; 110:2432–2439. [PubMed: 17606760]

Figure 1.

Isolation endothelial microparticles and study design. (A) A schematic of purification of endothelial cell microparticles and study design. (B) Electron microscopy imaging of EMPs after isolation. EMPs are variable in size with majority of them between 50 and 150 nm.

Figure 2. Endothelial microparticle uptake by disc annulus fibrosus cells. (A) A schematic of labeling of endothelial cells with a lipophilic membrane dye to produce green labeled microparticles. (B) Live cell imaging by confocal microscopy of annulus fibrosus cell (blue-nucleus, red-cytoplasm) at 0, 20, 40, and 60 min after being exposed to labeled EMP's (green). Images shown in anterior view (top) and in lateral view (bottom).

Figure 3.

Effects of endothelial microparticles and secreted soluble proteins on matrix and catabolic mRNA gene expression in annulus fibrosus cells. Untreated AF cells (CTL) and AF cells treated with endothelial microparticles (EMPs) or soluble proteins (SUP) as described in Figure 1A were analyzed by RT-PCR for mRNA expression of different MMPs (A), TIMPs (B), and the major disc matrix genes collagen type 1 and aggrecan (C). Each data point represents one individual sample and the bars represent the 95% confidence intervals. p-value was calculated using Mann–Whitney test where $p < 0.05$ is considered statically significant (*).

Figure 4.

Effects of endothelial microparticles and secreted soluble proteins on MMP and TIMP protein expression in annulus fibrosus cells. Conditioned culture media of untreated AF cells (CTL) and AF cells treated with endothelial microparticles (EMPs) or soluble proteins (SUP) as described in Figure 1A were analyzed by Western blots (A) to measure protein expression levels (B) of different MMPs and TIMPs. Each data point represents one individual sample and the bars represent the 95% confidence intervals. p-value was calculated using Mann–Whitney test where $p < 0.05$ is considered statically significant (*).

MMP Activity Assay

Figure 5. Effects of endothelial microparticles and secreted soluble proteins on MMP enzymatic activity in conditioned culture media of annulus fibrosus cells. MMP enzymatic activity was measured by a fluorescent assay in conditioned culture media of untreated AF cells (CTL) and AF cells treated with endothelial microparticles (EMPs) or soluble proteins (SUP) as described in Figure 1A. MMP activity of EMPs alone and SUP alone was also measured. p-value was calculated using Mann–Whitney test and $p < 0.05$ is considered statistically significant (*).

Figure 6.

Summary of study findings. Endothelial cells produce and secrete microparticles, some of which can be internalized into disc cells. Both endothelial microparticles (EMPs) and soluble protein factors induce disc cell catabolic activity and suppress key matrix gene expression such as aggrecan. Active MMPs were found only in EMP-treated AF cells conditioned media while inactive pro-MMPs were found in conditioned media of both EMP-treated and SUP-treated AF cell cultures, suggesting that EMPs play a pivotal role in activating MMPs

Table 1

Validated PCR primers, forward and reverse sequences respectively, of metalloproteinases (MMPs), collagen type 1 (Col-1), aggrecan (ACAN), tissue inhibitor of metalloproteinases (TIMPs), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Primers	Sequences (5'-3')
MMP-1	GAGCTCAACTTCCGGGTAGA (F) CCCCAAAAGCGTGTGACAGTA (R)
MMP-2	GCGCCGTCGCCCATCATCAA (F) AGCTCTCCTTGGGGCAGCCA (R)
MMP-3	CAAGGAGGCAGGCAAGACAGC (F) GCCACGCACAGCAACAGTAGG (R)
MMP-13	TGCTTCCTGATGACGATGTAC (F) TCCTCGGAGACTGGTAATGG (R)
Col-1	GCCTTCCTTGACATTGCTGAAGA (F) CTCCGTTGGACATAGAGAGGGTT
ACAN	AAGAATCAAGTGGAGCCGTGTGTC (F) TGAGACCTTGTCTGATAGGCACT (R)
TIMP-1	AGCAGAGCCTGCACCTGTGT (F) CCACAACTTGGCCCTGATG (R)
TIMP-2	GAATCGGTGAGGTCCTGTCCTGA (F) CCTGCACACAAGCCCGATAAA (R)
TIMP-3	TACCTGCCCTGCTTCGTG (F) AGGCGTAGTGTGTTGGACTGG (R)
GAPDH	ACCCACTCCTCCACCTTTGAC (F) TCCACCACCCTGTGCTGTAG (R)