INFLAMMATORY MICROENVIRONMENT ALTERS THE REGENERATIVE CAPACITY OF ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

Ph.D. THESIS

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UNIVERSITY OF SZEGED,

2024

SZEGED

SCIENTIFIC PAPERS INCLUDED IN THE THESIS

i. **Szűcs D**, Miklós V, Monostori T, Guba M, Kun-Varga A, Póliska S, Kis E, Bende B, Kemény L, Veréb Z. Effect of Inflammatory Microenvironment on the Regenerative Capacity of Adipose-Derived Mesenchymal Stem Cells. Cells. 2023 Jul 29;12(15):1966. doi: 10.3390/cells12151966. PMID: 37566046; PMCID: PMC10416993.; IF: 6.0; Journal specialization: *Scopus* – General Biochemistry, Genetics, and Molecular Biology, Location: *Q1*; *SJR*- Biochemistry, Genetics and Molecular Biology (miscellaneous)

ii. **Szűcs D,** Monostori T, Miklós V, Páhi ZG, Póliska S, Kemény L and Veréb Z (2024), Licensing effects of inflammatory factors and TLR ligands on the regenerative capacity of adipose-derived mesenchymal stem cells. Front. Cell Dev. Biol. 12:1367242. doi: 10.3389/fcell.2024.1367242; IF: 5.5; Journal specialization: Scopus -Cell Biology, Developmental Biology, Location: Q1; SJR-Developmental Biology

Total IF: 11.5

Publications not related to the thesis

I. Tamás Monostori, **Diána Szűcs**, Borbála Lovászi, Lajos Kemény, Zoltán Veréb. Advances in tissue engineering and 3D bioprinting for corneal regeneration. IJB null, 0(0), 1669. https://doi.org/10.36922/ijb.1669; IF: 7.422; (Journal specialization: *Scopus* Biotechnology; Location: *Q1*)

II. Kun-Varga A, Gubán B, Miklós V, Parvaneh S, Guba M, **Szűcs D**, Monostori T, Varga J, Varga Á, Rázga Z, Bata-Csörgő Z, Kemény L, Megyeri K, Veréb Z. Herpes Simplex Virus Infection Alters the Immunological Properties of Adipose-Tissue-Derived Mesenchymal-Stem Cells. Int J Mol Sci. 2023 Jul 26;24(15):11989. doi:

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III. **Szűcs D**, Fekete Z, Guba M, Kemény L, Jemnitz K, Kis E, Veréb Z. Toward better drug development: Three-dimensional bioprinting in toxicological research. Int J Bioprint. 2023 Jan 6;9(2):663. doi: 10.18063/ijb.v9i2.663.; IF: 7.422; (Journal specialization: *Scopus* Biotechnology, Industrial and Manufacturing Engineering,; Location: *Q1*)

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V. Guba M, **Szűcs D**, Kemény L, Veréb Z. Mesterséges bőrszövetek a kutatásban és a gyógyításban [Tissue engineered skin products in research and therapeutic applications]. Orv Hetil. 2022 Mar 6;163(10):375-385. Hungarian. doi: 10.1556/650.2022.32330. PMID: 35249001.; IF: 0.707

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Total IF: 30.966

1 INTRODUCTION

1.1 Wound healing

The process of wound healing is intricate and influenced by numerous factors, with chronic wounds requiring approximately 12 weeks for recovery due to underlying pathophysiological conditions. Effective healing relies on adequate blood circulation, as reduced oxygen and nutrient supply can impede the process. Venous leg ulcers, prevalent among individuals with chronic venous insufficiency, affect approximately 1.65-1.74% of adults aged 65 and older. Molecular mechanisms involved in wound healing include inflammation, proliferation, and tissue remodeling. Inflammation attracts immune cells to remove debris and fight infection, while growth factors stimulate tissue regeneration. Fibroblasts synthesize collagen, endothelial cells facilitate angiogenesis, and keratinocytes form a protective barrier. Tissue remodeling, guided by enzymes like MMPs, ensures proper wound closure. Disruption in these processes can lead to impaired healing and chronic wounds. A comprehensive understanding of these mechanisms is essential for developing effective therapeutic interventions.

1.2. Chronic wounds

Chronic wounds present a molecular complexity characterized by persistent inflammation, compromised cellular reactions, and abnormal extracellular matrix (ECM) remodeling. In contrast to acute wounds, which progress through healing stages promptly, chronic wounds exhibit prolonged inflammation characterized by elevated levels of pro-inflammatory cytokines and chemokines, disrupting the healing cascade. Dysfunctional cellular

responses, such as reduced fibroblast proliferation and ECM synthesis, along with impaired angiogenesis by endothelial cells, exacerbate tissue repair processes. Additionally, abnormal ECM remodeling compromises cell function and impedes tissue repair. A thorough understanding of these molecular mechanisms is imperative for the development of effective therapies aimed at addressing chronic wound healing.

1.3. Clinical therapy, regenerative medicine, and stem cells

Chronic wounds present formidable obstacles, requiring frequent medical attention for treatments like dressings, debridement, and compression therapy. These interventions often lead to hospitalization due to complications such as infections. Despite these efforts, healing can be prolonged or incomplete, with a notable recurrence of ulcers post-recovery, amplifying costs and the overall disease burden. Regenerative medicine, encompassing techniques like stem cell therapy, growth factors, and tissue-engineered products, offers hope in tackling these challenges. Mesenchymal stem cells (MSCs), sourced from various tissues, possess valuable traits like self-renewal, differentiation, and immunosuppression, making them versatile for therapeutic purposes. Adipose-derived MSCs (AD-MSCs), found in adipose tissue, exhibit multipotency and immunomodulatory properties, impacting immune responses and aiding in tissue repair. Despite their potential, ensuring the quality and effectiveness of AD-MSCs is crucial for their successful application. This study seeks to explore the wound healing and skin regeneration abilities of AD-MSCs in inflamed environments, aiming to enhance comprehension and therapeutic efficacy.

2. AIMS

Our research aims to trial a cell therapy method and study how adipose-derived mesenchymal stem cells (AD-MSCs) respond to inflammation in non-healing chronic wounds and ulcers. Stem cells must endure inflammation, proliferate, and use their regenerative and immunomodulatory abilities for tissue repair. Pretreating and licensing cells before therapy may improve efficacy, highlighting the importance of studying cellular functionality in both normal and inflammatory conditions before clinical use.

In the projects related to the thesis, our goal was to create an *in vitro* model of chronic non-healing ulcers to study the regenerative abilities of AD-MSCs.

1. Examine whether AD-MSC retains its tissue regenerative capacity, especially wound closure, in inflammatory microenvironments.
2. Define the MSCs secretome in the microenvironment typical of chronic non-healing
3. Analyze the gene expression pattern of the AD-MSC upon inflammatory conditions.
4. Investigate the gene expression changes for the biological pathways related to stemness and regenerative and immunological properties of the cells.
5. Identify new signaling processes and biological pathways that may be important for future therapeutic developments.
6. Build an extended model based on our previous results to study the effects of licensing on AD-MSC,

7. Employ a more sensitive cytokine detection method to measure difficult-to-detect, low-concentration cytokines.
8. Upgrade a more robust wound-healing assay.
9. Investigate the gene expression changes for the biological pathways related to stemness and regenerative and immunological properties of the cells.
10. Identify new signaling processes and biological pathways that may be important for future therapeutic developments.
11. Compare the gene expression changes caused by different treatments from a therapeutic point of view.

3. MATERIALS AND METHODS

3.1. Sample Collection

Adipose tissue collection adhered to the principles of the Declaration of Helsinki and was approved by the relevant regulatory bodies. Tissues were obtained from patients undergoing plastic surgery.

3.2. Stromal Vascular Fraction Isolation

Adipose tissue was isolated and processed to obtain the stromal vascular fraction (SVF), containing mesenchymal stem cells (MSCs). Following tissue digestion and centrifugation, the SVF was collected and suspended for subsequent experiments.

3.3. Mesenchymal Stem Cell Differentiation

AD-MSC differentiation was assessed using adipogenesis, osteogenesis, and chondrogenesis differentiation kits. Various staining methods confirmed differentiation into adipocytes, osteoblasts, and chondrocytes.

3.4. Surface Antigen Expression Analysis by Flow Cytometry

Surface antigen expression patterns of AD-MSCs were analyzed using flow cytometry with fluorochrome-conjugated antibodies.

3.5. Treatment of AD-MSC for RNA Isolation/Wound Healing Assay

AD-MSCs were treated with inflammatory agents, and subsequent RNA isolation or wound healing assays were performed.

3.6. Assessment of Cytotoxic Effects of Inflammatory Agents on AD-MSCs

The cytotoxic impact of inflammatory agents on AD-MSCs was evaluated using a cytotoxicity detection kit.

3.7. Cell Proliferation and Metabolism

Proliferation effects and changes in metabolism due to inflammation were assessed using BrdU incorporation and MTT assays, respectively.

3.8. Cellular Impedance Measurement

Label-free cellular impedance measurements were conducted using a real-time cell analysis instrument.

3.9. RNA Isolation for RNA Sequencing

Total RNA was isolated from cells using TRI Reagent® and subsequently processed for RNA sequencing.

3.10. RNA Sequencing

High-throughput mRNA sequencing was performed, and data analysis was conducted to obtain global transcriptome data.

3.11. RNA-Seq Analysis

Gene expression analysis was performed using R software, including differential expression analysis and gene set enrichment analysis.

3.12. RNA Isolation for qPCR

RNA isolation was conducted for quantitative PCR (qPCR) analysis.

3.13. RT-PCR

cDNA synthesis was performed to quantify gene expression levels.

3.14. qPCR

Quantitative PCR was conducted using specific probes to analyze gene expression.

3.15. Protein Array

Supernatants from treated AD-MSCs were analyzed using a protein array to determine secreted factors.

3.16. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to validate protein array results and quantify cytokine/chemokine levels.

3.17. Quanterix Multiplex Enzyme-Linked Immunosorbent Assay (ELISA)

Multiplex ELISA was employed to analyze cytokine levels in treated cells.

3.18. Wound Healing Assay

Various wound healing assays were conducted to assess the effects of inflammation on cell migration and wound closure. Microscopic imaging and impedance measurements were used for analysis.

3.19. Statistical analysis

The normality of data distribution was evaluated using Kolmogorov-Smirnov and Lilliefors tests. Each experiment was conducted at least three times, with each sample tested in triplicate. Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical significance was determined using two-way ANOVA analysis for comparisons involving more than two groups and paired Student t-test for comparisons between two

groups. A significance level of 0.95 was set, and p-values less than 0.05 ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) were considered significant.

4. RESULTS

4.1 Characterization of AD-MSCs and Differential Gene Expression

Primary AD-MSCs underwent trilineage differentiation and FACS analysis to confirm their identity. Treatment with LPS and TNFα led to significant alterations in gene expression profiles, with 2752 and 1613 DEGs identified, respectively. Both treatments influenced immunomodulatory gene expression, with TNFα showing a stronger effect on up-regulation. Chemokines and growth factors associated with wound healing processes were also affected by both treatments. Clustering analysis revealed distinct expression patterns between treatments and controls, with TNFα showing stronger effects on immune-related pathways.

4.2 Comprehensive Transcriptomic Profiling

The administered treatments exert a significant influence on gene expression, resulting in both upregulation and downregulation of numerous genes, with notable intersections. Downregulation was evident in the following manner: LPS (104 genes), TNF-α (87 genes), IL-1β (50 genes), IFN-γ (56 genes), and PolyI:C (83 genes), collectively affecting 25 genes. Conversely, upregulation was observed with LPS (14 genes), TNF-α (34 genes), IL-1β (26 genes), IFN-γ (38 genes), and PolyI:C (14 genes), impacting 109 shared genes. These findings underscore the intricate and multifaceted impact of the treatments on the transcriptomic profile, revealing both common and distinct regulatory responses.

4.3 ViSEAGO Analysis and Clustered Pathways

The analysis conducted using ViSEAGO reveals the presence of two primary treatment clusters: one comprising PolyI:C, TNF-α, and IFN-γ, and the other composed of IL-1β and LPS. These clusters exhibit variations in defense and immune response, signal transduction, and cellular processes, while sharing impacts on organelle organization and metabolic processes. Furthermore, each treatment uniquely influences specific pathways: LPS affects 2 pathways, TNFα impacts 8, IL-1β affects 1, IFN-γ affects 3, and PolyI:C affects 5 pathways. There are also shared alterations observed in multiple pathways, with LPS and TNFα affecting 4 common pathways, and IFNγ and PolyI:C impacting 3. Notably, TNF-α and IFN-γ cluster together in angiogenesis pathways. Additionally, TNF-α, IL-1β, and PolyI:C demonstrate similar effects in cell cycle pathways, while IFN-γ forms a distinct grouping. In terms of stem cell differentiation, LPS and IL-1β align closely with control samples, TNF-α clusters with PolyI:C, and IFN-γ forms an independent cluster. The varying effects of treatments on tissue regeneration and wound healing pathways underscore the nuanced and pathway-specific responses observed across different biological contexts.

4.4 Cellular Characterization and Safety Evaluation

AD-MSCs exhibited tri-lineage differentiation capacity and mesenchymal origin, ensuring their suitability for the study. Cytotoxicity and viability tests confirmed the safety of treatments on cellular viability and function.

4.5 Impact of Treatments on Wound Healing

Wound healing assays demonstrated accelerated wound closure in treated samples compared to controls, especially when treatments preceded wound induction.

4.6 Analysis of Gene and Protein Expression

The qPCR analysis unveiled distinct patterns of gene expression in response to the treatments. CXCL-8 demonstrated strong upregulation when exposed to LPS, TNFα, and IL-1β, while NAGS and STAT6 consistently displayed downregulation across all treatments. Unlike other treatments that led to a reduction in IL-6 mRNA levels, TNFα did not significantly impact IL-6 expression. CXCL-10 exhibited increased expression with TNFα treatment and a modest rise with IFNγ. ASGR1 showed decreased expression with LPS, TNFα, and IFNγ, but increased expression with IL1β and PolyI:C. ICAM1 experienced a slight increase with TNFα treatment, whereas it decreased with other treatments. At the protein level, IL-6 levels were elevated with LPS, TNFα, and IL-1β treatments, whereas IFNγ and Poly I:C treatments resulted in decreased levels. CXCL-8 increased with LPS and TNFα treatments but decreased with others, while CXCL-10 levels increased with all treatments, particularly with TNFα and IFNγ. The multiplex ELISA analysis revealed significant increases in IL-5, IL-12p70, and IL-22 following IL-1β treatment.

5. DISCUSSION

Mesenchymal stem cells (MSCs) offer promising prospects for therapeutic applications, yet optimizing their effectiveness remains a significant challenge. Previous studies indicate that MSC licensing can greatly improve tissue regeneration, reduce inflammation, and expedite wound healing. In our recent research, we focused on human adipose tissue-derived MSCs and exposed them to lipopolysaccharide (LPS) and tumor necrosis factor-alpha (TNFα) to mimic an inflammatory environment.

Through detailed analysis of gene and protein expression, we found compelling evidence of significant changes in MSC behavior.

Exposure to LPS and TNFα resulted in noticeable shifts in the transcriptomic and proteomic profiles of MSCs, particularly demonstrating increased expression of genes associated with crucial cellular processes such as proliferation, differentiation, and wound healing. Notably, TNFα treatment led to substantial upregulation of interleukins and growth factors, indicating a robust immune response. Conversely, LPS treatment primarily affected interleukin protein levels, highlighting the distinct effects of different inflammatory stimuli on MSC behavior.

Further investigation through STRING analysis revealed activation of key proteins involved in vascular remodeling, suggesting a potential role for MSCs in modulating the vascular microenvironment essential for wound healing. Moreover, both LPS and TNFα treatments activated proteins associated with MSC migration and immunosuppression, indicating their multifaceted impact on cellular behavior. Of particular interest is the observation that TNFα treatment induced a more pronounced immune response compared to LPS, indicating the emergence of a pro-inflammatory MSC phenotype. This finding suggests that the specific inflammatory environment to which MSCs are exposed can significantly influence their functional characteristics, thereby affecting their therapeutic potential.

While our findings provide compelling evidence of MSC activation's efficacy in promoting wound healing, numerous challenges and avenues for further exploration remain. Future research should focus on refining therapeutic protocols, optimizing cell delivery methods, and elucidating the mechanisms underlying MSC localization at injury sites. Additionally,

personalized treatments tailored to individual patients' unique molecular landscapes hold promise for enhancing therapeutic outcomes in the realm of chronic and inflamed wounds.

.6. SUMMARY

Our investigation delved into the impact of an inflammatory microenvironment on adipose-derived mesenchymal stem cells (AD-MSCs) for potential therapeutic applications. Treatment with inflammatory factors induced significant changes in gene expression, protein profiles, and wound closure rates. These findings highlight the clinical potential of AD-MSCs, particularly in addressing chronic inflammatory conditions. Understanding MSC dynamics in inflammatory environments offers insights for advancing regenerative medicine and personalized therapies.

ACKNOWLEDGMENT

I wish to extend my sincere gratitude to Prof. Dr. Lajos Kemény and Prof. Dr. Rolland Gyulai for granting me the opportunity to participate in their Ph.D. program and conduct my research in the laboratory of the Department of Dermatology and Allergology at the University of Szeged.

I am profoundly grateful to my supervisor, Dr. Zoltán Veréb, for his precious guidance and insightful advice throughout my doctoral studies.

I'm deeply thankful to Tamás Monostori for his significant contributions to experiments and statistical analysis in this project. Thanks for his uplifting chats and playful banter, which brought brightness to my day.

I am also thankful to Vanda Miklós for her contributions to sequencing data analysis and statistical support, which greatly enhanced the credibility of my research findings.

I express my heartfelt thanks to Katalin Boldog for her administrative and technical assistance during the research, as well as for her many positive and humorous thoughts that brightened up the everyday routine.

My appreciation extends to Dr. Szilárd Póliska for his expertise in performing RNA-sequencing, a pivotal aspect of our study.

Furthermore, I am grateful to Dr. Balázs Bende and Dr. Erika Kis for their involvement in procuring adipose tissue samples from patients.

I deeply appreciate the support and assistance provided by all members of the Department of Dermatology and Allergology throughout my Ph.D. journey.

Finally, and most importantly, I wish to convey my genuine appreciation, deep gratitude, and admiration to my love, family, and friends. Their unwavering love has provided me with stability during challenging times and celebrated alongside me in moments of joy.