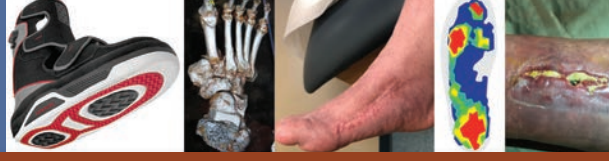

Extracellular Vesicles in Diabetic Wound Healing

These secretions show great potential for healing DFUs.

BY TIMOTHY CHEUNG, DPM, PHD, CPT AND STEPHANIE WU, DPM, MSC

Diabetic Foot Ulcers

Diabetic foot ulcers affect millions of people in the United States and worldwide, and the incidence of diabetes mellitus continues to rise. Approximately 537 million adults, aged 20–79 years, worldwide suffer from diabetes, and the number is projected to rise by over 11% to 643 million by 2030, and to 783 million by 2045.[1] The pathophysiology of diabetic foot ulcers is complex and its effects can be devastating. Diabetic foot ulcer is a major risk factor for developing infections, subsequent hospitalizations and amputations[2-5] and it has been estimated that a diabetic amputation takes place every 30 seconds around the world.[6] Moreover, management of the diabetic foot and its complications remain a high economic burden on the healthcare system[7-9], not to mention the deleterious psychosocial effects on the patient's quality of life because of impaired mobility and substantial loss of productivity.[3] The risk of developing foot ulcers in patients with diabetes is up to 25%.[10]

Although the biggest risk factor for developing a diabetic foot ulcer is a previous foot ulcer, developing new technologies to accelerate the diabetic foot ulcer into remission can alleviate much of the health and financial burden placed by the diabetic foot.[11] Furthermore, if the wound has not healed more than 50% at the four-week mark with standard of care, an alternative or additional treatment approach should be considered, which may include new wound care technology.[12] Advances in technology have resulted in numerous wound care products commercially available to help facilitate the healing process. Exosomes and extracellular vesicles comprise an emerging new wound care technology that shows promise in the field of diabetic wound healing.

What Are Exosomes and Extracellular Vesicles?

Extracellular vesicle is defined by the International Society for Extracellular Vesicles as "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e. they do not contain a functional nucleus."[13] All cells release extracellular vesicles, some as part of normal physiology while others as an adaptive response. There are two types of extracellular vesicles: exosomes and ectosomes. Ectosomes are vesicles that bud outwardly from the plasma membrane which produce microvesicles, microparticles, and large vesicles. In contrast, exosomes originate from endosomal invagination. Exosomes are generated by sequential invagination of the plasma membrane, forming multivesicular bodies that contain intraluminal vesicles within the cell. The multivesicular bodies fuse with the plasma membrane, releasing the intraluminal vesicles as exosomes.[14]

Although both types of extracellular vesicles may have diagnostic and therapeutic potential, we focus on exosomes in this review, as evidence emerges regarding their efficacy in diabetic wound healing.

Exosomes were first discovered in mammalian red blood cells in 1983;[15-17] and the term "exosome" was coined in 1987 to describe this heterogeneous population of lipid bilayer membrane-enclosed vesicles. At the time, these extracellular vesicles were thought of as a process to selectively remove certain plasma membrane proteins during their maturation process. Essentially, exosomes were thought of as a "garbage disposal" mechanism, posing limited therapeutic potential. In 2007, a group of researchers discovered that exosomes contained mRNA and microRNA, important regulators of cellular processes.[18] Since then, other studies have revealed exosomes to contain retrotransposon RNA transcripts,

Advances in technology have resulted in numerous wound care products commercially available to help facilitate the healing process.

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oncogene amplifications, mitochondrial DNA, and single- and double-stranded DNA.[19-21]

These works suggest that exosomes may have a higher function than transporting cellular garbage. Rather, they may function as a mode of intercellular communication, transporting and delivering bioactive cargos, such as proteins, lipids, nucleic acids, long noncoding RNAs, and multi-molecular complexes, further mediating cell-to-cell communication, and regulating physiological processes such as metabolism and homeostasis.[22]

People with different diseases release exosomes with different RNA and protein contents into the circulation that can be measured as biomarkers. The discovery of exosomes as natural carriers of functional small RNA and proteins has led to an explosion of interest in the cell-derived biotherapeutics and drug delivery field, as it may be possible to harness these vesicles for therapeutic delivery of microRNA, peptides, and synthetic drugs.

Exosomes and extracellular vesicles have emerged as promising diagnostic and therapeutic tools for many different disease processes. Exosomes can be found in all biological fluids including blood, saliva, semen, lymph fluid, breast milk, urine, amniotic fluid, and cerebrospinal fluid[23] and are secreted by all cells. Therefore, non-invasive liquid biopsies remain a viable option. Recent technological advances have resulted in the emergence of a variety of extracellular vesicle isolation methods; and the diagnostic application of exosomes has focused on diseases of the central nervous system,[24] cardiovascular diseases,[25-26] and cancer[27-28], and show promise in diseases involving the kidney,[29] lung[30] and liver.[31]

It is critical to recognize that exosome isolation methods are still being refined and current biomarkers may only identify subpopulations of exosomes.[13,32-34] Moreover, the International Society for Extracellular Vesicles calls for a tighter regulation for the nomenclature.[35] Therefore, whenever the definition of exosomes is unclear in the literature, we will refer to them as extracellular vesicles.

Exosomes can be found in all biological fluids including blood, saliva, semen, lymph fluid, breast milk, urine, amniotic fluid, and cerebrospinal fluid[23] and are secreted by all cells.

Extracellular Vesicles in Mesenchymal Stem Cells

There is a growing interest in mesenchymal stem cell (MSC)-derived extracellular vesicles in regenerative medicine.[36] One study sought to understand the role of MSCs in diabetic wound healing.[37] In this study the investigators grafted MSCs extracted from human bone marrow into wounds of healthy and diabetic mice. Each animal had two separate wounds. Interestingly, both normal and impaired mice given MSCs healed quickly, irrespective of whether the wounds directly received MSC grafts, reflective of a systemic response to MSC engraftment.

Furthermore, endogenous MSCs from the host were mobilized to the wound in both normal and impaired healing animals. This work suggests that the key to wound healing does not hinge on exogenous MSC targeting, but rather the recruitment of host MSCs. Moreover, it is not dependent on where you place the MSCs, but rather how the MSCs stimulate their resident counterparts already in the body.

Therefore, there is potential for therapeutic discovery in identifying the signaling mechanisms MSCs use to trigger the body's own stem cells to heal, such as exosomes and extracellular vesicles. Extracellular vesicles derived from MSCs have been noted in studies to mainly act via their encapsulated microRNAs, a class of non-coding RNAs that play important roles in regulating gene expression. However, other studies have shown that MSC derived extracellular vesicles can act independently of microRNAs.[22]

There is a growing interest in mesenchymal stem cell (MSC)-derived extracellular vesicles in regenerative medicine.[36]

Recent animal models demonstrated that extracellular vesicles from pig adipose tissue-derived stem cells mainly participate in endothelial cell migration, extracellular matrix remodeling, blood coagulation, inflammation, and angiogenesis.[38] Bone marrow stem cell derived extracellular vesicles primarily promote cell proliferation and viability.[39]

Exosomes Derived from Pre-treated MSCs

Not all exosomes are created equally. They vary in size, content, localization, and function depending on the cell types they are derived from.[14] Adipose tissue-derived stem cells, for example, has been found to secrete a much higher amount of extracellular vesicles compared to other cell types.[22] While exosomes and extracellular vesicles have properties similar to their parental cells, they are often excreted based on the surrounding conditions. It is therefore reasonable to wonder if pre-treatment of MSCs would potentially change exosome outcomes. One group of researchers pre-treated MSCs with

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atorvastatin, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor.[40]

Another group pre-treated MSCs with pioglitazone, a peroxisome proliferator-activated receptor, activator (PPAR, activator), which is a common drug used to treat diabetes mellitus.[41] Multiple studies have shown that exosomes derived from MSC pre-treated with hypoxic conditions yielded exosomes with better therapeutic potential.[42-43] Therefore, a third group of researchers pre-treated MSCs with deferoxamine, an agent that mimics the effects of hypoxia.[44] The findings from all three groups of researchers noted that exosomes isolated from pre-treated MSCs resulted in increased angiogenesis and healing rates at diabetic wound sites.

Indeed, there are many avenues in which exosomes can be used. Along with isolating exosomes and extracellular vesicles from pre-treated MSC populations, it is also possible to isolate exosomes from other cell populations. In a study by Shi and colleagues, their group purified a CD63, CD9 and ALG-2-interacting protein X (Alix) positive exosome population from activated platelets and applied them in a rabbit ischemic wound healing model.[45]

These researchers noted that the activated platelet-derived exosomes promoted full-thickness healing with reconstitution of hair follicles and sebaceous glands through delivery of transforming growth factor-beta (TGF-beta). Moreover, they used an in vitro model to demonstrate cell proliferation, migration, and skin organoid formation, consistent with the notion that these TGF-beta-loaded exosomes are capable of promoting revascularization and collagen synthesis, even in the presence of ischemia.

Alginate-based hydrogels, especially, have received recent attention because of their biocompatibility and capacity for sustained release of their carried extracellular vesicles.[66]

The Role of Exosomes on Macrophage Polarization and Wound Healing

Wound healing and tissue regeneration are a complicated series of biochemical processes that create an orderly healing cascade with four key phases: hemostasis, inflammation, proliferation, and remodeling.[46] Impaired macrophagic activity has been implicated in patients with diabetes.[47-51]

Beyond the traditional role of clearing wound debris and bioburden, macrophage polarization into different M1-like and M2-like phenotypes plays an important function in determining the chronicity of wounds.[52-54] Briefly, M1-like phenotypes assist in the traditional paradigm of phagocytic macrophages that clear wound debris and bioburden and act in the pro-inflammatory phase while polarization into an M2-like phenotype plays an important role in the repair phase, including collagen deposition and angiogenesis.[55-56]

Exosomes secreted by adipose tissue-derived stem cells, for example, induce the polarization of macrophages to the M2 phenotype, thus reducing the ability of macrophages to stimulate the inflammatory response.[57] Extracellular vesicles from diabetic patients were also noted to be preferentially internalized by circulating monocytes.[58] Co-incubation of diabetic extracellular vesicles with monocytes reduced the expression of genes related to apoptosis and oxidative stress in monocytes, and increased expression of pro-inflammatory cytokines.[58]

These data suggest that extracellular vesicles in diabetic patients promote monocyte survival and promote monocyte inflammatory activation. It is therefore reasonable to ask whether there will be a different response to exogenous extracellular vesicles. Indeed, investigators found that treatment with exogenous exosomes up-regulated M2-associated genes, which were associated with faster healing rates of diabetic full-thickness wounds.[60]

Macrophage-derived exosomes provided therapeutic activity by inhibiting the secretion of pro-inflammatory cytokines.[59]

Furthermore, macrophage-derived exosomes provided therapeutic activity by inhibiting the secretion of pro-inflammatory cytokines.[59] Based on available data, targeting abnormal exosome internalization may be a promising avenue in treating diabetic wounds.

Extracellular Vesicles-Loaded Scaffolds

Although exosomes and extracellular vesicles have high therapeutic potential, their pharmacokinetic delivery can be challenging. Exosomes and extracellular vesicles can be delivered by systemic injection or topical application. When injected systemically through direct intravenous, intraperitoneal or subcutaneous injections, the exosomes were rapidly cleared from blood circulation and accumulated in the liver, spleen, lung, and gastrointestinal tract in as quickly as two hours after injection.[61-62]

The majority of these exosomes are rapidly cleared by macrophages in the reticuloendothelial system and excreted.[63-64] Topical exosomes can be difficult to directly apply on wounds for healing due to their rapid clearance by fluids

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and exposure to external elements.[65] Instead, exosomes can be encapsulated into scaffolds such as biodegradable or highly porous hydrogels, that keep the extracellular vesicles at the wound site to deliver sustained therapeutic effects. They can also help prevent the exosomes from being cleared prematurely and allow the delivery of a more concentrated and localized exosome dosage by placing the hydrogel directly at the wound site.[65]

Alginate-based hydrogels, especially, have received recent attention because of their biocompatibility and capacity for sustained release of their carried extracellular vesicles.[66] A study by Wang and colleagues demonstrated that the application of exogenous exosomes using exosome-laden self-healing injectable hydrogel accelerated diabetic full-thickness wound healing, worked even faster than exosomes alone.[60] Interestingly, further analysis revealed that the accelerated wound healing was due to M2 macrophage induction and enhanced angiogenesis to the wound site.

Exosome and extracellular vesicle therapy in pre-clinical studies have also demonstrated immunomodulatory and reparative properties that can potentially accelerate diabetic wound healing.[22]

Logistical Advantages of Exosomes and Extracellular Vesicles

Cellular-based therapy holds a promising avenue for treating diabetic foot ulcers. Of these, stem cell therapy has gained particular traction.[67-68] There are a few logistical advantages to using exosomes and extracellular vesicles compared to stem cell therapy including their status as mediators of cell-to-cell communication via transfer of biological cargo, and their reported ability to cross biological barriers that impede many delivery systems.[69]

Extracellular vesicles generally have good stability because of the double lipid membranes that can protect internal biomolecules from various enzymes in body fluids, thereby maintaining their integrity and biological activity.[22] Exosomes are stable at room temperature and can be stored longer-term at temperatures of 4, -20 or -80 degree Celsius.[69] Since there is no living tissue applied to the body, there are fewer apparent restrictions by regulatory bodies when using exosome therapy compared to cell-based therapies.

Pre-clinical research indicates that exosomes and extracellular vesicles may help accelerate diabetic ulcer healing due to their immunomodulatory and reparative properties to promote tissue regeneration and repair by regulating cell proliferation, angiogenesis, apoptosis, and extracellular matrix remodeling.[22] Moreover, outcomes of exosome therapies in pre-clinical research appear to be comparable to cell-based therapies. One in-vivo diabetic wound model study evaluated the efficacy of extracellular vesicles-loaded oxygen releasing antioxidant wound dressing and noted improved angiogenesis, faster re-epithelialization, enhanced collagen synthesis, and accelerated wound repair.[70]

Another pre-clinical study found dressing containing exosomes and silver nanoparticles demonstrated broad-spectrum antimicrobial activity and promoted wound healing.[71]

Conclusion

The incidence of diabetes mellitus has been steadily increasing worldwide and foot ulcers are a common, serious, and costly complication of this devastating disease. Treatment of the diabetic foot ulcer is challenging and numerous wound care products are commercially available to healthcare providers to help facilitate healing. Exosomes and extracellular vesicles are considered a new generation of a natural nanoscale delivery system, an emerging new wound care technology that shows promise in the field of diabetic wound healing.

Exosomes and extracellular vesicles are secretions by different types of cells that allow for intercellular communication.

Exosomes and extracellular vesicles are secretions by different types of cells that allow for intercellular communication. These modes of communication may act as a vehicle that carry different signal molecules such as RNAs and proteins that can provide diagnostic, targeted drug delivery, and therapy potential. Exosomes and extracellular vesicles have been useful in the diagnosis of various diseases including cardiovascular diseases, central nervous system disorders, and certain cancers. There is high therapeutic potential of exosomes since they are efficient at entering cells and can deliver functional cargo with minimal clearance by the immune system, in contrast to liposomes.[72-74]

Because of this, there are numerous studies that investigate the clinical utility of exosomes and extracellular vesicles in various disease states.[14] Exosome and extracellular vesicle therapy in pre-clinical studies have also demonstrated immunomodulatory and reparative properties that can potentially accelerate diabetic wound healing.[22]

Although much of this research has shown positive results, further investigation is required to solidify its translation into human medicine, specifically in the setting of treating diabetic foot ulcers. **PM**

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