The Exosome - A Naturally Secreted Nanoparticle and its Application to Wound Healing

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Wound healing is a complex process and often delayed in patients with underlying chronic conditions. The cost of wound care is a significant burden to the society, warranting new techniques to prompt wound healing. Several studies have reported on the beneficial effects of mesenchymal stem cells (MSCs) function in recruiting host cells, releasing secretory factors and matrix proteins thereby increasing wound heal. These secrete bioactive trophic factors from MSCs also includes extracellular vesicles (EVs) or exosomes. Recent studies have shown that EVs are one of the key secretory products of MSCs mediating cell-to-cell communication to enhance wound healing. Current knowledge related to the potential use of EVs in wound healing is reviewed and the promising future for EVs - a naturally secreted nanoparticle - as an alternative to cell-based therapy is discussed.

1. Introduction

The skin is the largest organ in the human body and its main function is to protect the body from invading pathogens. However, the skin is frequently injured by acute and chronic wounds caused by diabetic skin ulcerations or extensive burns. It not only causes physical and mental suffering in affected individuals, but also imposes a huge socioeconomic burden. Thus, numerous on-going investigations are focusing on expediting the wound healing process. In recent years there have been advances in stem cell transplantation therapy, tissue engineering, exploring the role of microRNA in tissue regeneration and also the role of extracellular vesicles (EVs) in healing of wound. In this article, we review current knowledge and treatment related to wound healing and the potential use of EVs to enhance healing of the wound.

2. Extracellular Vesicles (EVs)

Small vesicles were reported to be secreted by sheep reticulocytes in 1980 by Pan and Johnstone[1] and the term "exosomes" was coined in 1987 by Johnstone et al.[2] **Table 1** details the history of discovery of exosomes. However, there are different subtypes of vesicles with overlapping composition, density, and size.[3] Due to the lack of specific markers for different subtypes, the International Society of Extracellular Vesicles suggested the term extracellular vesicles (EVs) to describe the preparations of vesicles from body fluids and cell cultures.[3,4] The term EV broadly includes exosomes, microvesicles, prostasomes, ectosomes, and oncosomes (**Figure 1**).

The biogenesis of exosomes starts with the early endosomes where endocytic vesicles incorporate their content intended for recycling, degradation, or exocytosis. The early endosome is then transformed into the late endosome where contents are sorted into 30–100 nm vesicles that bud into the lumen of late endosomes or with intraluminal vesicles (ILV).[5] The late endosomes either fuse with lysosomes to destroy the content of late endosome or the plasma membrane, resulting in the secretion of the 30–100 nm vesicles into the extra-cellular milieu.[3,6]

Microvesicles (MVs), also referred to as shedding MVs, are large membranous vesicles (50–1000 nm diameter) that are produced by budding from the plasma membrane. The molecular mechanism of exosome and MV biogenesis shares a lot of similar elements.[7]

Prostasomes are submicron membranous vesicles, secreted by the human prostate gland into prostatic fluid and range between 40–500 nm diameters.[8] Prostasomes are found to influence and modify the characteristics of sperm.[8,9] However, these prostasomes are found to turn against their host after 50 years of age increasing the prevalence of prostate cancer.[9]

Ectosomes are extracellular vesicles generated by outward budding from the plasma membrane. Unlike exosomes, ectosomes do not require exocytosis instead it pinches off from the plasma membrane and releases to the extracellular space. These vesicles are 100–350 nm in diameter.[10] As other EVs, ectosomes are being studied as a target for new therapies. Le et al. (2014) proposed that ectosomes play an important role in cancer metastasis by transferring miR-200s from high metastatic cells to poor metastatic cells.[11] Ectosomes are believed to promote inflammation and cell death in rheumatoid arthritis and multiple sclerosis.[10]

Oncosomes, as the name suggests, are membranous microvesicles containing an active oncogene or oncogenic

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factor. Oncosomes are secreted from various cancer cells such as prostate cancer cells, human brain tumors etc., facilitating intercellular transfer of oncogenic information.[12,13] These oncosomes are also capable of altering the tumor microenvironment and promoting disease progression.[13]

2.1. Composition of EVs

EVs play an important role in cell-to-cell communication and cell-cell interaction. EVs are not only involved in normal physiological processes but also play an important role in the development and progression of diseases.[3] The cargo content of EVs depends on the source of the cell type. EVs cargo consists of proteins, microRNA (miRNA), mRNA and lipids. The details of the proteins, RNA and lipid content of the EVs can be obtained from ExoCarta,[14] Vesiclepedia[15] and EVpedia.[16] A total of 538 studies (as of July 2015) have been submitted to Vesiclepedia – an exosome database.[16] Valadi et al. (2007) were the first to report that EV cargo consists of mRNAs and miRNAs.[17] Since then there has been 27,642 mRNA and 4,934 miRNA entries in the Vesiclepedia database. Interestingly, it has also been reported that gene transcripts are exclusively carried by EVs, which are undetectable in donor cells.[18]

EVs, being of endosomal origin are known to carry membrane transport proteins, proteins involved in multivesicular body (MVB) biogenesis (Alix, TSG101), fusion proteins including GTPases, Annexins, flotillin and rab proteins, which may promote the fusion of MVB with the cell membrane. EVs are also known to carry tetraspannins (CD9, CD63, CD81 and CD82), adhesion molecules (CD11b and CD54) and heat shock proteins (heat shock cognate 70 and heat shock proteins 90; indicative of cellular response to environmental stresses). EVs also carry lipid-related proteins including cholesterol, sphingomyelin and ganglioside GM3[19] and externalized phosphatidylserine.[20] Both lipid-related proteins

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Table 1. History timeline for the discovery and applications of EVs.

Year	Application	Discovery	Ref
1977	Prostatic and seminal fluids	Extracellular presence of vesicles surrounded by a bilayer membrane	[70]
1980	Sheep reticulocytes	Vesicle formation	[1]
1987	Sheep reticulocytes	Coined the term exosome	[2]
1996	Human and murine B lymphocytes	Adaptive immune response	[71]
1998	Mouse dendritic cell	Promote induction of antitumor responses in mice	[72]
1999	Mouse dendritic cell	Characterized the composition and physical properties of EVs	[73]
2007	Mouse and human mast cell line, primary bone marrow-derived mouse mast cells	Contain messenger RNA and microRNA	[17]
2009	Different cell types	Database of RNA, protein and lipids	[74]
2008	Glioblastoma tumor cells	Promote tumor progression	[75]
2010	Leishmania donovani	Immunosuppressive contributing to the tolerance of an invader	[76]
2011	Raji B-cell line, Jurkat-derived J77 T cell line, and primary dendritic cells	Modulates interactions between antigen-presenting cells and T cells	[77]
2011	Self-derived dendritic cells	Generated targeted-EV by engineering the dendritic cells to express Lamp2-fused to the neuron-specific RVG peptide	[63]

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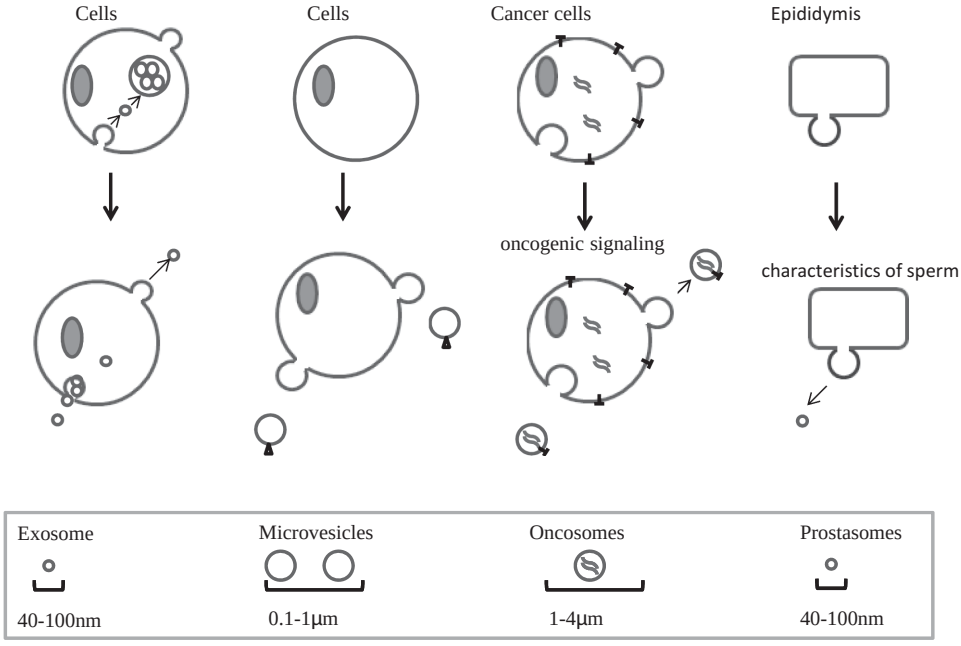

Figure 1. Schematic representation of major populations of extracellular vesicles. Left – Exosomes are the EVs of endosomal origin and formed by the inward budding of the inner membrane of the cells. Microvesicle originates from outward budding and fission of plasma membrane. Oncosomes are generated from plasma membrane blebs. Prostasomes shed from the epididymal plasma membrane in an apocrine fashion.

and phospholipases play an important role in vesicle structure and function, and trafficking of particular proteins to EVs.[21] Packaging of RNA into EVs are selective and consist of microRNAs (miRNAs), small non-coding RNAs (piRNA, snRNA, snoRNA, scaRNA, Y RNA), natural antisense RNAs, tRNAs and their fragments, mRNAs and their fragments, rRNAs, and long non-coding RNAs.[22] However, a major limiting factor in the field of EV cargo analysis is the use of range of different isolation and characterization methods between different labs.

2.2. Internalization of EVs

The recipient cells take up EVs via direct membrane fusion, endocytosis or cell-type specific phagocytosis. More importantly, these EVs are well capable of delivering their content of miRNA and mRNA into the target cells, affecting their microenvironment as well as modulating their protein production.[23] Studying the internalization of EVs in detail, Feng et al. (2010) reported that EVs (derived from chronic myelogenous leukemia (K562) or human T-cell lines (MT4)) are internalized more efficiently by phagocytic cells (RAW 264.7 macrophages, U937 monocyte-derived macrophages, THP-1 myelomonocytic cells and J774A.1 macrophages) than non-phagocytic cells (Jurkat T cells, 293T cells, COS-7 kidney cells, HEL299 human lung fibroblasts, or mouse fibroblast NIH 3T3 cells).[24] Using live-cell imaging Feng et al. (2010) showed that non-phagocytic cells cannot internalize EVs efficiently but they remain associated with their cell membranes.[24] EV internalization depends on several factors including lipid composition and temperature, as it is best at physiological temperature.[25] It has also been shown that EV internalization in human metastatic melanoma cells is increased at acidic pH, which also increases its release. In particular the acidic tumor microenvironment might play a role in increased EV uptake by the tumor cells.[25]

2.3. Isolation of EVs

EVs can be isolated from a wide range of body fluids (including blood, urine, bronchoalveolar lavage fluid, breast milk, amniotic fluid, synovial fluid, pleural effusions, and ascites) and also from cell culture supernatants (including T-cells, B-cells, dendritic cells, platelets, mast cells, epithelial cells, endothelial cells, neuronal cells, cancerous cells and mesenchymal stem cells (MSCs)).[3] The most widely cited purification method is a series of high-speed centrifugation (≈ 100 000 ×g) to isolate EVs either from body fluids or cell culture supernatant.[26,27] However, there are several commercially available kits used for isolating EVs both from body fluids and cell culture supernatant, which are less labor-intensive. Invitrogen Total Exosome Isolation Kit (Life Technologies, USA) uses low-speed centrifugation (10 000 ×g for 60 min), whereas Exo-spin (Cell Guidance Systems, USA) columns for purification of EVs. ExoQuick (System Biosciences (SBI), USA) purifies EVs by precipitating them from body fluids and cell culture supernatant.

Lamparski et al. (2002), isolated EVs from the media conditioned by monocyte-derived dendritic cells (MDDCs). The conditioned media was clarified through a 3/0.8 μm Sartoclean CA filter (500 cm^2) to remove cells and cell debris and concentrated by ultrafiltration through a 500 kDa MWCO hollow fiber membrane (UFP-500-C-4A; lumen diameter of 0.5 mm; surface area of 650 cm^2) maintaining a transmembrane pressure of 4–7 psi. The conditioned media was further

concentrated to 75–100 ml followed by diafiltration (five times with an equal volume of DPBS (i.e., 5 × 75–100 ml)) using the same hollow fiber cartridge. Following this the conditioned media was centrifuged (4× ≈25 ml) (Beckman) and underlayed with a density cushion composed of 20 mM Tris/30% sucrose/deuterium oxide (D_2O) pH 7.4 (4 ml) forming a visible interphase. The samples were then ultracentrifuged at 100 000 ×*g* and 4 °C for 75 min (Beckman). The EV–sucrose density cushions was then pooled and diafiltered into formulation buffer (20 mM Tris/1 mM $MgCl_2$/5% sucrose/100 µg ml^{-1} human serum albumin pH 7.4) through a 500-kDa miniature hollow fiber cartridge (UFP-500-C-MM01A) as above. Finally, EVs were sterile filtered through a 0.22 µm Sterivex-GV capsule filter prior to use in clinical setting.[28]

The other method described by Lamparski et al. (2002), was miniscale purification method. This method included removal of dead or non-adherent cells from media conditioned by dendritic cells by either filtration through 0.8 µm filter or by centrifugation at 1000 ×*g* for 10 min. This conditioned media was then concentrated by centrifugation for 30 min at 1000 ×*g* in a prerinsed 100 kDa MWCO Centricon Plus-80 capsule filter to a volume of 1–2 ml. Concentrated EVs were collected and resuspended in 70 ml DPBS, then centrifuged at 1000 ×*g* for 30–60 min (volume remained was <1.8 ml), followed by inverting the sample tube and recentrifuging for an additional 1 min to isolate EVs. This isolated EVs was ultracentrifuged, underlayed with 300 µl of a 30% sucrose/D_2O density cushion at 100 000 ×*g* and 4 °C for 40 min. Isolated EVs were then diluted in DPBS and concentrated by centrifuging for 30–60 min at 1000 ×*g* in a prerinsed 100 kDa MWCO Millipore Ultrafree-15 capsule filter to a desired volume.[28]

2.4. Characterization of EVs

There are different ways to characterize the EVs isolated from cell culture supernatant or bodily fluids. The most important characteristics studied are size and protein markers. The size of the EVs can be determined using transmission electron microscopy (TEM), scanning electron microscopy (SEM), and nanoparticle tracking analysis (NTA).[29] For detecting some of the classical biomarkers (frequently used – TSG101, CD63 and ALIX), immunogold labelling of EVs can be done before visualizing using TEM.[26,30] Immunoblotting is the convenient method to characterize the EVs using EV-specific protein markers. EVs are too small to be analyzed using flow cytometry therefore, it has to be fixed to the beads of a size that is in the detection range of a flow cytometer before labelling with fluorophore-conjugated antibodies and analyzing by FACS (Fluorescence-activated cell sorting).[30]

3. Mesenchymal Stem (Stromal) Cells

Due to the high rate of mortality and morbidity associated with wounds, new approaches are being explored to accelerate the healing of wounds. There is mounting evidence in the literature, which shows the association of mesenchymal stem (stromal) cells (MSCs) with tissue repair and wound healing.[3,31] Initial studies reported that the therapeutic potency of MSCs was due to the fact that they easily differentiate into various cell types such as chondrocytes, adipocytes, osteoblasts or endothelial cells. However, it has now emerged that MSCs may predominantly act in a paracrine fashion and secretory factors being the mediators of tissue repair and wound healing.[3,32] EVs are one of the many secretory factors released by the MSCs. EVs derived from MSCs as well as many other cell lines are known to show therapeutic potential and is detailed in **Table 2**.

3.1. Wound Healing: Some Statistics

Wound healing is a coordinated effort of different tissues and cell lineages to replace/repair the missing cellular structures and tissue layers. Most of the wounds heal without problems but healing can be delayed in any patient with underlying illness. Moreover, chronic wounds may take longer to heal, sometimes months or years, or may never heal. Chronic wounds (in the case of diabetes) not only lead to increasing healthcare costs but also adversely affect the individual's quality of life.

About 382 million diabetes patients are registered globally and this number will rise to 592 million by 2035.[33] Approximately 15% of the patients develop diabetic foot ulcer that frequently become infected in an estimated 2 million patients.[34] The annual cost of caring for diabetic foot ulcer patients is estimated $5 billion in US alone. The wound healing society reported about 15% of older adults in the US suffer from chronic wounds, including predominantly venous pressure ulcers (bedsores), stasis ulcers, and diabetic (neuropathic) foot ulcers and the incidence increases every year with 2 to 3 million more diagnosed with various types of chronic wounds.[35] Another cause of wound is domestic or work related burns. Nearly 10 million people are burned every year worldwide and require medical attention (WHO, Fact sheet N°365).[36] Burn leads to temporary or permanent disability exceeding billions of dollars as direct costs for care.

3.2. Wound Healing Phases

Healing of wound includes four phases - haemostasis, inflammation, proliferation, and tissue remodelling or resolution.[37–39] In **Table 3** and **Figure 2** we have summarized the mediators, target cells and their effects at different phases of wound healing.

Haemostasis: Haemostasis in the wound takes place immediately after occurrence of wound and vasoconstriction and coagulation commences essentially to prevent exsanguination by platelet aggregation and clot formation providing matrix for the other host cells required for healing.[40] The blood clot also traps platelets, growth factors and cytokines attracting neutrophils, macrophages, endothelial cells and fibroblasts.[41] The blood vessels then dilate allowing the influx of more thrombocytes and other blood cells.

Inflammation: As the skin is damaged there is a loss of barrier or protection against invading micro-organisms, the inflammation phase creates an immune barrier by initiating a series of molecular events. Neutrophils, monocytes and macrophages

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Table 2. Summarizing therapeutic potential of EVs.

Type of EV	Host cell	Disease model	Mode of application	Results	Ref
Engineered EVs- expressing transmembrane domain of platelet-derived growth factor receptor fused to GE11 peptide	Human embryonic kidney (HEK) cell line 293	Xenograft breast cancer tissue in RAG2$^{-/-}$ mice	Intravenous injection	Effectively target EGFR-expressing cancerous tissues with nucleic acid drugs	[64]
Engineered EVs- expressing suicide gene mRNA and protein–cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT)	HEK-293T	Nerve sheath tumors in athymic mice	Intratumoral injection	Effectively deliver therapeutic mRNA/proteins to treatment of diseases	[78]
Exosome-mimetic nanovesicles loaded with doxorubicin	Human umbilical vein endothelial cells and CT26 colorectal carcinoma cells	Malignant tumor - BALB/c mice bearing subcutaneously transplanted CT26 cells	Intravenous injection	Effectively deliver chemotherapeutics to treat malignant tumors	[79]
Exosome-like nanoparticles	Grapes	Dextran sulfate sodium - induced colitis mice	Oral	Modulate intestinal tissue renewal processes and remodelling in response to pathological triggers	[80]
Exosome expressing Tspan8 at a high and CD49d at a medium level (LnStr-α4)	Lymph node stroma line	BDX rats	Intravenous injection	Dye-labeled LnStr-α4-exosomes binds more efficiently to peritoneal exudate cells, kidney and pancreas	[81]
Exosomes encapsulated curcumin	EL-4 murine lymphoma cells	Lipopolysaccharide (LPS)-induced brain inflammation model, experimental autoimmune encephalitis and a GL26 brain tumor model	Intranasal non-invasive	Protected from LPS-induced brain inflammation, the progression of myelin oligodendrocyte glycoprotein peptide induced experimental autoimmune encephalomyelitis, and had significantly delayed brain tumor growth in the GL26 tumor model	[82]
Exosomes encapsulated curcumin	EL-4 (mouse lymphoma cell line)	Lipopolysaccharide (LPS)-induced septic shock mouse model	Intraperitoneal injection	Enhanced anti-inflammatory activity of curcumin	[83]
Exosome-like nanovesicles + miR-150	T cells	Trinitrophenyl- or Oxazolone sensitized mice	Intraperitoneal, intravenous and oral	Exosome-like nanovesicles deliver inhibitory miRNA to target effector T cells in an antigen-specific manner by a surface coating of antibody light chains	[84]
miR-146-expressing exosome	Mesenchymal stem cells	Fischer rats xenografted intracranially with 2.5×10^5 9L gliosarcoma cells	Intratumoral injection	Significantly reduced glioma xenograft growth in a rat model of primary brain tumor	[85]
Folate receptor-α-positive exosomes	Z310 choroid plexus epithelial cells	Healthy C57BL/6	Intraventricular injections	Efficient exosome-mediated folate delivery into the brain parenchyma	[86]
miR-143 overexpressing exosome	HEK-293 cells and PNT-2 prostate cells	BALB/c mice -PC-3M cells injected subcutaneously	Intratumoral injection	Secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process	[87]
GDNF incorporated exosomes	Raw 264.7 macrophages	6-OHDA-intoxicated BALB/C mice	Intravenous injection	Attenuate and reverse progression of Parkinson's disease	[88]
EV	Mesenchymal stem cells	Focal cerebral ischemia in C57BL6 mice	Intravenous injection	Attenuates the effects of focal cerebral ischemia	[89]
Exosome	Induced pluripotent stem cells-MSCs	Mice with femoral artery excision	Injected into left quadriceps muscle	Attenuate limb ischemia and promote angiogenesis	[90]
Exosomes/microvesicles	Mouse cardiac fibroblast (CF)-derived iPS cells	Ischemic myocardium mice	Intramyocardial injection	Effective at transmitting cytoprotective signals to cardiomyocytes	[91]
Exosome	Neuroblastoma	Immunosuppressed mice with tumor (CHLA-255 and human monocytes) and transfected with miR-155	NA	Role of exosomic miR-21 and miR-155 in the cross-talk between NBL cells and human monocytes in the resistance to chemotherapy	[92]
Exosome	Blood of adult rats and human volunteers	Ischemia-reperfusion Sprague Dawley rats	Intravenous tail vein injection	Activate cardioprotective pathways	[93]

Table 3. Summary of the mediators, target cells and their effects at different phases of wound healing.

Wound healing phase	Mediators	Target	Effects	Ref
Haemostasis/Coagulation	Platelet-derived growth factor (PDGF); transforming growth factors A1 and 2 (TGF-A1 and TGF-2); epidermal growth factor, insulin-like growth factors	Endothelial cells, Platelets	Blanching, Clot formation	[40,46,94]
Inflammation	TGF-β, tumor necrosis factor-α (TNF-α), heparin binding epidermal growth factor, fibroblast growth factor (FGF), interleukin-1 (IL-1)	Activating keratinocytes, endothelial cells, leukocytes, neutrophils, macrophages	Phagocytosis -removes foreign material, bacteria, dead cells, and damaged ECM	[40,95]
Proliferation	Interleukins, FGFs, TNF-α vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), TGF-β, tissue inhibitor of metalloproteinases (TIMP) vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), and TGF-β, PDGF, angiogenin	Fibroblasts	Matrix production, Angiogenesis, Collagen synthesis	[45,95]
Remodelling	PDGF, TGF-β, α–smooth muscle actin, TIMPs, and FGFs	Myofibroblasts, fibroblasts, Endothelial cells	"switching" of fibroblasts to myofibroblasts	[39,96,97]

are attracted to the wound site within 24 – 72 hours after injury. Phagocytosis is induced by neutrophils to remove bacteria, foreign particles and damaged tissue.[42,43] After completing the task, neutrophils undergo apoptosis followed by phagocytosis by macrophages to eliminate apoptotic bodies and cell remnants.[43,44] Macrophages also act as a key regulatory cell in activating keratinocytes, fibroblasts, and endothelial cells.[40] If the microbial clearance is not complete it can elongate the inflammatory phase and the wound may enter a chronic state, failing to heal.[37]

Proliferation: The inflammatory phase is followed by the proliferative phase. On day three fibroblasts appear and start proliferating and producing matrix proteins including hyaluronan, fibronectin, proteoglycans and type 1 and type 3 procollagen. Collagen plays an important role in proliferative and remodelling phase of wound repair process providing a foundation to the matrix formation. Epidermal cells, fibroblasts, macrophages, and vascular endothelial cells also initiate angiogenesis forming microvascular network.[45] During the proliferation stage, epithelial cells can also be found to start migrating towards the wounded area from the edges.[46]

Remodelling: The final phase of wound healing is remodelling. It starts 4 or 5 days after injury and lasts for more than 2 weeks, depending on the wound-healing environment. Collagen is constantly degraded and extracellular matrix is remodeled to provide strength to the wound and decrease wound thickness.[46] The underlying connective tissue shrinks in size and brings the wound margins closer together.

3.3. MSCs in Wound Healing – Clinical Studies

In the first randomized trial Dash et al. (2009) recruited patients with chronic non-healing ulcers including diabetic foot ulcers and Buerger disease and treated them with autologous bone marrow-derived MSCs (BM-MSC). Intramuscular or subcutaneous injection of BM-MSC showed a significant decrease in ulcer size.[47] Earlier, a study carried out on patients with chronic cutaneous ulcerations, analyzed healing of wound by directly applying fresh autologous bone marrow aspirate as well as injecting into the edges of the wound. Additionally, these patients were also treated with cultured bone marrow cells applied topically to the wound.[48] Topically applied bone marrow cells lead to both dermal rebuilding and reduced scarring and ultimately resulting in closure of non-healing chronic wounds.[48]

Topical application of autologous MSCs was also studied in patients with acute wounds (n = 5) due to skin cancer surgery and chronic, long-standing, non-healing lower extremity wounds (n = 8).[49] Cultured autologous MSCs were applied onto the wounds using a fibrin polymer spray system that resulted in decreased chronic wound size.[49] Authors also reported that topical application of autologous MSCs resulted in closure of full-thickness wounds in diabetic mice.[49]

4. Chronic Wound Treatment Using EVs

4.1. Proliferation/Growth

To understand the effect of EVs on healing of wound, Zhang et al. (2014) established a rat second-degree burn injury model. Authors reported significantly enhanced re-epithelialisation of the wound, when treated with both human umbilical cord MSC (hucMSC)-derived EVs (200 µg) and hucMSC (1×10^6 cells suspended in 200 µl PBS) compared to human lung fibroblasts (HFL1) or HFL1-Ex groups when injected subcutaneously at three sites.[50] Increased proliferation of skin cells was also found to be associated with increased level of cytokeratin 19 (CK19) and proliferating cell nuclear antigen (PCNA). It was found that hucMSC-EVs inhibited heat stress-induced apoptosis in HaCAT (keratinocytes cells) and DFL (Dermal fibroblasts) cells by reducing the level of Bax, a pro-apoptotic protein.[50]

Increased migration of fibroblasts was observed in the presence of both 50 µg ml^{-1} and 100 µg ml^{-1} hiPSC-MSC-EVs at 12 and 24 hours compared to control.[51] Zhang et al. (2015) also found increased migration in HUVECs using both 50 µg ml^{-1} and 100 µg ml^{-1} hiPSC-MSC-EVs.

Shabbir et al. (2015) studied the effect of EVs on normal fibroblast cells and reported a significant and dose-dependent

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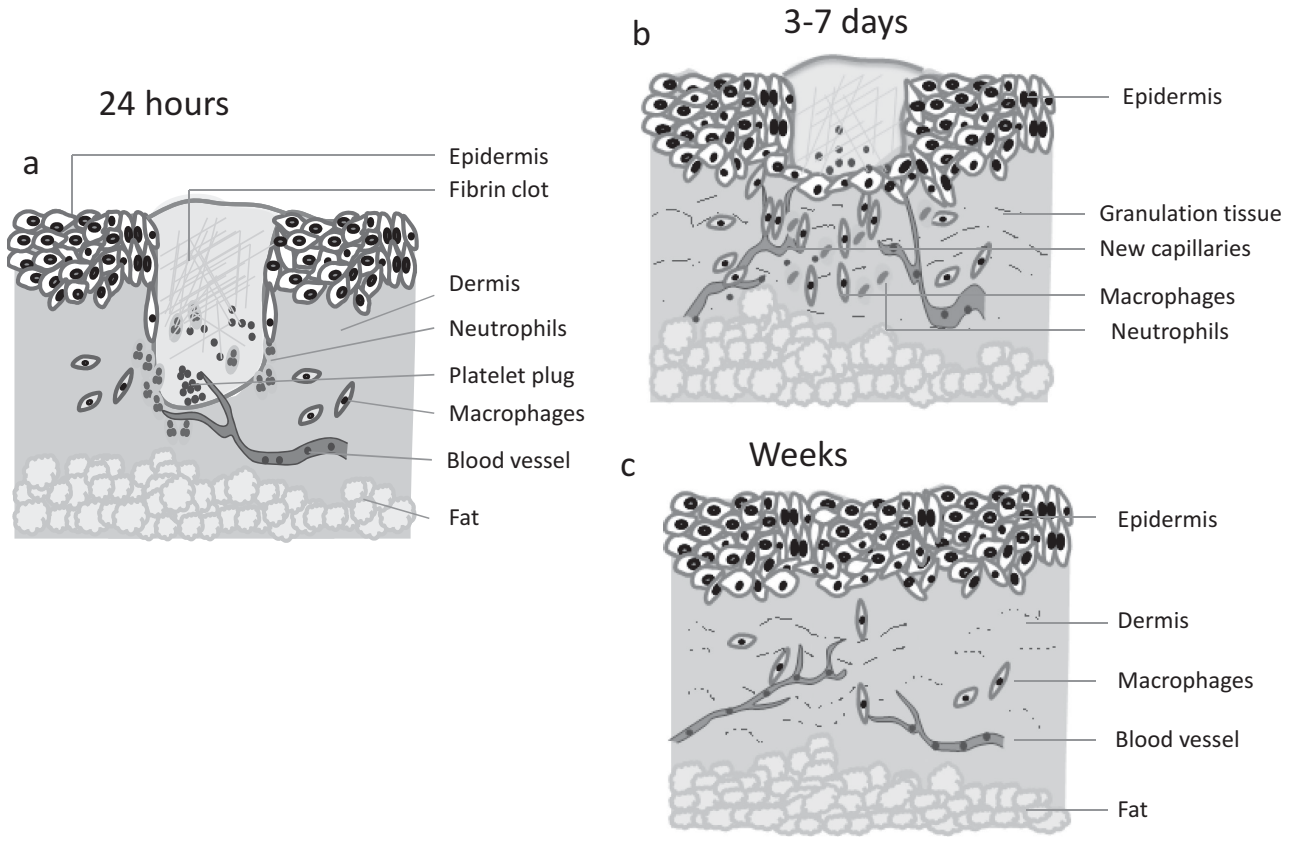

Figure 2. Stages of acute wound healing response under normal physiologic conditions. a) Blood clotting, platelet aggregation, and migration of neutrophils and macrophages to the site of injury is initiated due to injury. Within 24 hours, a blood clot is initiated that is composed of fibrin and fibronectin providing a scaffold for cell migration and aggregated platelets and are known to secrete growth factors into the surrounding tissue. b) By 3 days numerous neutrophils and macrophages are recruited to the site of injury, phagocytosing and killing microorganisms as well as producing growth factors into the wound environment. By 5 days tissue granulation starts that is composed of fibroblasts, additional macrophages, and neovasculature. The last phase includes epidermal migration and ingrowths of granulation tissue.

increase in growth when treated with MSC-EVs. Conversely, Shabbir et al. (2015) also reported that the growth of diabetic wound fibroblasts was significantly diminished when treated with MSC-EVs.[52] When studying the rate of migration in normal and diabetic wound fibroblasts, the authors observed enhanced migration in both normal and diabetic wound fibroblasts when treated with MSC-EVs.[52]

Timely and efficient repairing of intestinal mucosal wound and resolving inflammation, is critical in maintaining mucosal homeostasis. EVs isolated from intestinal epithelial cells (IECs) showed that annexin A1 (ANXA1) is released as one of its EV-content. These ANXA1-containing EVs were associated with significantly enhanced wound repair in IEC cells. ANXA1 enriched EVs were also isolated from leukocytes after injury, and were found to orchestrate epithelial wound repair.[53] Human induced pluripotent stem cell-derived mesenchymal stem cells (hiPSC-MSCs)-derived EV increased proliferation of both fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) in a dose dependent manner (using 50 μg ml^{-1} and 100 μg ml^{-1}), probably by inducing the protein expression of fibronectin, Type I, III collagen and elastin.[51] Further, authors also observed reduced scar widths with increased collagen maturity in a rat skin wound model treated with hiPSC-MSC-EVs compared to either controls or untreated groups.[51]

4.2. Angiogenesis

One of the important components of wound healing is formation of new blood vessels. There are several studies reporting the function of EVs in increasing angiogenesis in several wound heal models. The study of tubule formation in HUVECs, after treating with 100 μg ml^{-1} hiPSC-MSC-EVs, revealed enhanced tube formation at 4, 6, and 18 hours compared to control cells.[51] MSC-EVs were also found to enhance tubule formation compared with controls (vehicle and depleted medium) at all dosages. Depleted medium had a small, yet statistically significant, ability to induce tubular formation compared with vehicle.[52]

As $CD34^+$ cells were found to be significantly inducing angiogenesis in ischemic tissue, Sahoo et al. (2011), further explored the effect $CD34^+$ EVs on angiogenesis. Length of the tubule formed was significantly greater in HUVECs incubated with $CD34^+$ cell-CM or with $CD34^+$ EVs than in HUVECs incubated

with PBS.[54] No tubule formation was observed when HUVECs were incubated with the EV-depleted CM. Although the underlying mechanism of enhanced angiogenesis is not fully understood Sahoo et al. (2011) determined that $CD34^+$-EVs are enriched with pro-angiogenic microRNAs including miR-126 and miR-130a.[54]

4.3. Mechanisms of Wound Healing

The mechanism of wound healing might depend on the origin of EVs. Here we discuss the mechanism of action of EVs, derived from different cell lines, in wound healing.

β-catenin signaling plays a significant role in skin development as well as cutaneous wound healing. Zhang et al. (2015) studied the expression of Wnt family members and found that Wnt4 was significantly higher in hucMSCs compared to HFL1 cells and was also confirmed in hucMSC-Ex. The authors further confirmed that hucMSC-Ex-mediated Wnt4 activated β-catenin and thereby enhanced wound healing.[50] Investigating the mechanism of hucMSC-EV in wound healing, Zhang et al. (2015) proposed that EVs delivered Wnt4 to activate Wnt/β-catenin in skin cells, thereby inhibiting acute heat stress-induced skin cell apoptosis via AKT pathway activation.[50] Another study by Zhang et al. (2015), reported β-catenin nuclear translocation was promoted by hucMSC-Ex that induces the expression of proliferating cell nuclear antigen, cyclin D3, N-cadherin, and β-catenin and decreases the expression of E-cadherin.[55] β-catenin activation in endothelial cells induced by hucMSC-Ex-mediated Wnt4 increases proangiogenic effects thereby enhancing cutaneous wound healing.[55]

MSC-EVs was also found to activate several intracellular signaling pathways including AKT, ERK, and STAT3 and also induce expression of cell cycle genes and growth factors (including hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), and stromal-derived growth factor-1 (SDF1)).[52] STAT3 signaling plays an important role in cellular proliferation, migration, and angiogenesis mediating normal wound healing and response to injury.[56] STAT3 also regulates cell cycle control (c-myc and cyclin) and encodes cytokines and growth factors (IL-6, HGF, and VEGF).[52,57]

4.4. Engineering EVs

EVs are naturally secreted vesicles playing a crucial role in the horizontal transfer of RNA to neighboring or distant recipient cells. To harness this property, EVs can also be further engineered for targeted up take by the specific cells. It has been proven that RNAi delivery has therapeutic potential in several diseases including cancer, Parkinson's disease and HIV infections.[58] RNAi therapy and liposomal drug delivery holds great potential but their successful implementation is hampered by low bioavailability, inability to cross the blood-brain-barrier (BBB), non-internalization,[59] premature contents leakage[33,60] and acute immunological reaction.[61,62] Apparent lack of RNAi therapy in clinical trials could be attributed to unapproved clinical delivery system.[62] EVs are able to overcome the conventional barriers of drug delivery systems. EVs are internalized by fusing through the cell membrane.[60] As EVs are naturally produced nanovesicles it is complimented with reduced toxicity and immune response with the ability to cross BBB.[63]

Alvarez-Erviti et al. (2011), used EVs from self-derived dendritic cells to deliver short interfering (si)RNA to the brain in mice. Dendritic cells were engineered to express an EV membrane protein Lamp2b, fused to the neuron-specific rabies viral glycoprotein (RVG peptide) to generate targeting EVs.[63] Beta-secretase 1 specific siRNA was loaded in the targeting EVs using electroporation and was administered to normal C57BL/6 mice, resulting in significant decrease in BACE1 mRNA levels.[63]

Another study reported the efficient delivery of microRNA (miRNA) to epidermal growth factor receptor (EGFR)-expressing breast cancer cells. Targeting was achieved by engineering the human embryonic kidney cell line 293 (HEK293) cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide.[64] HEK293 cells expressing GE11 were further transfected with synthetic Let-7a before isolating EVs. Tumor-bearing (xenograft HCC70 cells) mice were injected with Let-7a–containing GE11-positive EVs and control EVs via the tail vein.[64] The authors reported suppressed tumor growth in Let-7a–containing GE11-positive EVs compared to control EVs.[64]

Tian et al. (2014) used murine immature dendritic cells (imDCs) to isolate EVs to reduce immunogenicity and toxicity. imDCs were further engineered using membrane protein Lamp2b fused to αv integrin-specific iRGD peptide to facilitate tumor targeting. EVs were loaded with doxorubicin (Dox) using electroporation.[65] Tian et al. (2014) reported that targeting EVs were efficiently able to deliver Dox to αv integrin-positive breast cancer cells in vitro. When intravenously injected these targeted EVs efficiently delivered Dox, specifically to tumor tissues, inhibiting tumor growth without apparent toxicity.[65]

There have been several studies showing expression of targeting peptides on the surface of the EVs, which could be degraded during biogenesis by endosomal proteases. To prevent these targeting peptides from degrading, Hung et al. (2015) proposed to include glycosylation motif at various positions.[66] This glycosylation motif not only prevented degradation of the target peptides but also increased their expression.

4.5. EVs in Clinical Trials

A phase I clinical trial for immunization of stage III/IV melanoma patients evaluated EVs purified autologous monocyte derived- dendritic cell (DC) cultures.[67] Fifteen patients were recruited (with criteria - stage IIIB and IV, HLA-$A1^+$, or -$B35^+$ and HLA-$DPO4^+$ leukocyte phenotype, tumor expressing MAGE3 antigen) from year 2000–2002 and received four EV vaccinations. This trial reported tumor shrinkage in one patient, minor response in one patient and stabilization in two patients highlighting the safety of EV administration.[67]

A similar phase I study enrolled HLA A2+ patients with pretreated Stage IIIb (n = 4) and IV (n = 9) non-small cell lung cancer with tumor expression of MAGE-A3 or MAGE-A4.[68] EVs were purified from autologous DC pulsed with the MAGE

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tumor antigens. This clinical trial also concluded that EV based vaccine is feasible and well tolerated in patients with advanced NSCLC.[68]

Yet another clinical trial was conducted for colorectal cancer treatments using ascites-derived exosomes (Aex) in combination with the granulocyte-macrophage colony-stimulating factor (GM-CSF). A total of 40 patients (HLA-A0201(+)CEA(+)) with advanced colorectal cancer were recruited and randomly assigned to treatments with Aex alone or Aex plus GM-CSF.[69] This clinical trial reported that Aex plus GM-CSF but not Aex alone induced beneficial tumor-specific antitumor cytotoxic T lymphocyte response. In this study the authors suggested that immunotherapy of colorectal cancer using Aex combined with GM-CSF is feasible and safe.[69]

5. Conclusion

EVs portray a promising future in providing cell-free therapy for several diseases requiring regeneration of tissues. Based on the extensive review of current publications, here we hypothesize that EVs derived from stem cells could be the next "off-the-shelf" product for wound healing. EVs are proven to contribute to an important mechanism for cutaneous wound healing including proangiogenic effects, activating wound-healing pathways as well as promoting growth factor secretion and collagen synthesis. EVs are naturally produced nano-vesicles and are complimented with both reduced toxicity and immune response with the ability to cross the cell membrane. EVs can be used as a nanoparticle and can be modified for targeted therapy. It can also be used as nanovehicle to transfer RNAi therapy as well as drugs to the target cells reducing the toxicity.

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