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Electrospun bioactive mats enriched with Ca-polyphosphate/retinol nanospheres as potential wound dressing

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ABSTRACT

Background: While electrospun materials have been frequently used in tissue engineering no wound dressings exist that significantly improved wound healing effectively.
Methods: We succeeded to fabricate three-dimensional (3D) electrospun poly(D,L-lactide) (PLA) fiber mats into which nanospheres, formed from amorphous calcium polyphosphate (polyP) nanoparticles (NP) and encapsulated retinol ("retinol/aCa-polyP-NS" nanospheres [NS]), had been incorporated.
Results: Experiments with MC3T3-E1 cells revealed that co-incubation of the cells with Ca-polyP together with retinol (or incubation with retinol/aCa-polyP-NS) resulted in a significant synergistic effect on cell growth compared with particle-free polyP complexed with Ca^{2+} or amorphous Ca-polyP NPs and retinol alone. Incubation of the cells in the presence of the retinol/aCa-polyP NSs also caused a significant increase of the expression levels of the genes encoding for the fatty acid binding protein 4 (FABP4), as well as of the genes encoding for leptin and the leptin receptor. In contrast, the single components, soluble Na-polyP, complexed to Ca^{2+}, or retinol-free aCa-polyP NPs, and retinol, had no significant effect on the expression of these genes.
Conclusions: These results indicate that the PLA fibers, supplemented with aCa-polyP-NP or retinol/aCa-polyP-NS, elicit morphogenetic activity, suggesting that these fiber mats, along with the antibacterial effect of polyP, have a beneficial potential as wound dressings combining antimicrobial and regenerative (wound healing) properties.
General significance: The PLA-based fiber mats, containing retinol and polyP nanoparticles, provide promising bioactive meshes that are urgently needed as dressings for chronic wounds.

1. Introduction

Wound healing is a complex and sequential, biological process that involves at least four consecutive but overlapping and highly programmed phases (reviewed in: [1]): Hemostasis, inflammation, proliferation, and remodeling. First, during the hemostasis period that immediately starts after wound setting vascular constriction and fibrin clot formation occurs. Second after bleeding is under control, pro-inflammatory cytokines and growth factors, including platelet-derived growth factor, fibroblast growth factor and epidermal growth factor, are released. Parallel with these events, a sequential infiltration of neutrophils, macrophages and lymphocytes, as well as platelets takes place that contributes to the prevention of blood loss [2]. The function of these cells is the elimination of invading microorganisms as well as the removal of cellular debris within the damaged tissue region. These cells produce and release protease(s) and reactive oxygen species, which might cause bystander damage, but also morphogenetically active polymers, *e.g.* polyphosphate (polyP) from platelets [3]. Third, during granulation tissue or new stroma formation, especially during the early phase of cutaneous wound repair, new capillaries endowing the neostroma with its granular appearance and macrophages, fibroblasts, and blood vessels move into the wound [4]. Finally, fourth, remodeling occurs that results in scar formation. Especially, during this phase the interaction of the cells, involved in remodeling, requires, for functional regeneration, the synthesis of the extracellular matrix by fibroblasts [5].

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Both local and systemic factors are involved in wound healing. Major local factors that directly influence wound healing include (*i*) oxygenation/superoxide radical formation, involved in energy production and/or oxidative killing of pathogens, (*ii*) infections *via* microorganisms which are normally restricted to the skin surface but might invade the underlying tissue strata. While inflammation is a physiological period in the course of the wound-healing process, during which contaminating microorganisms are eliminated, a prolonged inflammation period caused by bacterial endotoxins and mediated by pro-inflammatory cytokines, *e.g.* interleukin-1 and tumor necrosis factor-α, may lead to an extended or even chronic inflammatory status. The main determining systemic factors affecting wound healing are the age status of the patient which results in a temporal delay in wound healing [6]. The major alterations are delayed T-cell migration into the damaged wound area, reduced macrophage phagocytic activity and delayed re-epithelialization as well as collagen synthesis. Likewise, the shift in the balance between female estrogens (estrone and 17β-estradiol) and male androgens (testosterone and 5α-dihydrotestosterone) during aging adversely affect wound healing [7]. In addition, physical and psychological stresses that are associated with cardiovascular disease or cancer compromise wound healing. Furthermore, the impaired healing in patients with diabetes involving both hypoxia and dysfunction in fibroblasts and other epidermal cells impairs angiogenesis and neo-vascularization (reviewed in: [1]).

The well established and described medications of wound healing involve glucocorticoid steroid drugs, non-steroidal anti-inflammatory compounds or even chemotherapeutics. Adversely determining factors during wound healing are distinct, unhealthy lifestyle habits, like obesity, excessive alcohol consumption or smoking [8]. Wound healing supportive nutrients, like poly-unsaturated fatty acids, positively influence the production of the essential or pro-inflammatory cytokines or vitamins; *e.g.*, retinol and vitamin C show potent anti-oxidant, anti-inflammatory and, in turn, pro-wound healing effects [9].

As mentioned above, during wound healing cytokines and growth factors are released that initiate and maintain the interaction between the cells and control the regenerative response of the infiltrating cells. Besides of those soluble compounds external factors are efficient as well as regeneration-promoting stimuli. Among those is retinoic acid and its precursor retinol. Retinoic acid causes a differential gene expression by activating the genes controlling the pathways involved in retinoic acid esterification, synthesis from its precursor(s), and metabolism [10]; simultaneously, retinoic acid down-regulates the expression of the genes encoding for lipid metabolism during keratinocyte differentiation. In spite of an increased fibroblastic proliferation potency elicited by retinoic acid, the production of collagen was found to be diminished. In contrast to retinoic acid retinol has been proven to improve significantly wound healing [11].

It is well established that retinol is metabolically transformed into retinoic acid, especially *in vivo* by an enzymatically mediated two-step conversion *via* retinal and causes a differential gene expression within the retinoic acid nuclear receptors complex [12]. In response cellular proliferation and differentiation is modulated. However, only a small fraction of topically applied retinol is metabolized to retinoic acid, while it undergoes an alternative conversion into a series of metabolites, *e.g.* 14-hydroxy-retro-retinol which is involved in regulation of cell growth and death of lymphocytes. Finally, retinol was found to produce reactive oxygen species that activate different protein kinase pathways likewise involved in the control of morphologic and proliferative alterations in some human cells [13].

A new aspect in wound healing *via* restoration of endothelial progenitor cell functions is the finding that leptin, administered systemically and topically improves re-epithelialization of wounds in mice [14]. In addition, these authors described that keratinocytes, located at the wound margins, express the leptin-receptor subtype ObRb during the repair phase. Furthermore, leptin elicits a mitogenic stimulus to human keratinocyte cells *in vitro*. Those data are strongly corroborated by the finding that *ob*/*ob* (leptin null), and *db*/*db* (leptin receptor null), mouse strains show severe deficiencies in cutaneous wound repair [15]. Interestingly enough leptin primarily released from adipocytes but also synthesized in placenta, ovaries or skeletal muscle regulates fat deposition in the body (reviewed in: [16]). The leptin receptor has been originally identified in the hypothalamus. Applying both the techniques of polymerase chain reaction (PCR) and *in situ* hybridization evidence could be presented in a mouse model that leptin expression is acutely up-regulated in experimental wounds [17], suggesting that leptin is acutely up-regulated in injured skin tissue. Since also the leptin receptor is expressed in those cells autocrine/paracrine regulatory signaling cycle(s) have been proposed [17].

It is the aim of the present study to fabricate three-dimensional (3D) porous mats comprising loosely connected fibers with high porosity and high surface area by electrospinning. This widely used technology utilizes electrical forces to produce polymer fibers with diameters ranging from 2 nm to several micrometers and using polymer solutions of both natural and synthetic origin (reviewed in: [18]). This technology offers the favorable potential to synthesize novel natural nanofibers comprising controllable pore structures. As recently outlined only a few wound dressings have been developed that combine both prevention of microbial infiltration and stimulating wound cell regeneration [9]. In the present study we have developed wound covering meshes/mats that combine the property to allow a gas exchange environment with antibacterial properties as well as with a morphogenetic activity for the cells involved in wound healing. We succeeded to encapsulate retinol into nanospheres [19], formed from polyP nanoparticles, and fabricated them into electrospun fibers made of poly(D,L-lactide) (PLA). It is shown in the present study, that those mats retain the morphogenetic activity, to increase the expression of the fatty acid binding protein 4 (FABP4) as well as of leptin and its receptor, determined to be elicited by the retinol/polyP nanospheres that are not embedded into PLA. In previous studies it has been determined that polyP comprises also an antibacterial activity [20] and hemostatic activity [20].

For the administration of polyP and retinol to the cells had been fabricated into nanospheres; polyP acted as a bioscaffold for the production of nanospheres into which retinol has been encapsulated to form the nanospheres. Those polyP nanoparticles had been fabricated as amorphous particles; in those state, polyP maintained its morphogenetic activity and can be taken up by endocytosis [19]. For the studies here osteoblast-like MC3T3-E1 cells have been used that had been implicated in wound healing as well [21].

2. Material and methods

2.1. Materials

Na-polyphosphate (Na-polyP) with an average chain length of 30 phosphate units $(NaPO_3)_{n=30}$ was obtained from Merck Millipore (#106529; Darmstadt; Germany), all-trans retinol (#95144; $\geq$ 97.5%, M_r 286.45) was from Sigma (Taufkirchen; Germany).

2.2. Cultivation of MC3T3-E1 cells

The mouse calvaria cells MC3T3-E1 cells (ATCC-CRL-2593; #99072810; Sigma) were cultivated in a-MEM (Gibco-Invitrogen,