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Review

Role of stratifin in wound healing

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ABSTRACT

14-3-3 is a highly conserved, ubiquitously expressed protein family, and consists of seven mammalian isoforms (α, β, σ, δ, ε, η, γ, τ, ζ). Since their initial discovery in 1967, the 14-3-3 proteins have gained special importance as regulators in crucial biological processes such as cell cycle control, signal transduction, apoptosis, neuronal development and malignant transformation. Emerging evidence has shown that 14-3-3 sigma, also called stratifin, is unique among the isoforms due to its preference for homodimerization and specific selectivity for target binding proteins, and because it is most abundantly expressed in epithelial cells. Upon binding to the aminopeptidase N/CD13 receptor, stratifin stimulates the expression of matrix metalloproteinases (MMPs) and modulates extracellular matrix turnover which is a key determinant for successful wound healing. The identification of aminopeptidase N (APN) as a candidate receptor for the stratifin-mediated MMP-1 up-regulation in fibroblasts extends the functionality of the stratifin protein to cell migration and adhesion, immune response, differentiation, and metastasis. Further characterization of the stratifin/ APN-mediated signalling will provide valuable information needed for deciphering the intricacy of tissue regeneration and wound repair. In this review, the recent finding on the role of stratifin as a signalling factor in epidermal-dermal crosstalk and its receptor will be outlined and discussed.

KEYWORDS: wound healing, stratifin, amino-peptidase N

ABBREVIATIONS

MMP, matrix metalloproteinase; ECM, extra-cellular matrix; APN, aminopeptidase N; FN, fibronectin; TN-C, tenascin-C

INTRODUCTION

The 14-3-3 proteins function as molecular chaperons and serve as regulators in various biological processes. The seven mammalian isoforms of the 14-3-3 family (α, β, σ, δ, ε, η, γ, τ, ζ) are highly conserved proteins, and share 50% identity in amino acid sequences [1]. They can form homodimers or heterodimers [2]. Despite the high sequence similarities, comprehensive analysis of 14-3-3 interactomes showed that there is only 25% overlap among their target proteins [3-5]. The interactions appear to be isoform-specific, implying that these isoforms perform independent functions/roles in the body. 14-3-3 sigma (also known as stratifin) is unique among the isoforms due to its binding specificities and preference to homodimerize [6], and because it is primarily expressed in epithelial cells [7]. The importance of stratifin is further emphasized by the wide variety of pathological conditions involved [8-10], and loss of stratifin function is an indication of tumour development [11]. In this review, we will discuss the role of keratinocyte releasable stratifin as a signalling molecule and its influence on the

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modulation of extracellular matrix proteins by dermal fibroblasts. In particular, we will discuss the recent identification of its receptor, amino-peptidase N/CD13, as well as the therapeutic potentials of targeting stratifin or aminopeptidase N (APN) in wound repair.

Overview of wound healing

Within minutes of injury, platelets aggregate at the injured site to form a fibrin clot and this so called “hemostasis” is an initial event of the intricately orchestrated process of wound healing. Then, neutrophils and macrophages appear to phagocytose bacteria and tissue debris. In response to cytokines and growth factors secreted by macrophages [12], circulating mesenchymal progenitor cells (fibrocytes) extravasate into the wound site. Concurrent with the accumulation of fibroblasts (known as “fibroplasia”), endothelial cells are attracted into the wound bed by fibronectin in the clot, and together they engage in the formation of granulation tissue which is characterized by new blood vessels and provisional matrix. Keratinocytes from the wound edges migrate over the newly formed granulation tissue until they encounter migrating cells from the opposite direction. Meanwhile, circulating fibrocytes differentiate into myofibroblasts [13] which then contract to pull on the surrounding ECM, thereby reducing wound size. The wound undergoes constant alterations in ECM composition, in particular, collagen deposition and reorganization.

Epidermal-dermal communication

The healing of wounds requires intricate harmonization of many different cell types, such as immune cells, fibroblasts, keratinocytes, and endothelial cells within the wound healing environment [14-16]. These cellular interactions are mediated through the release of cytokines or via direct gap junctions. For many years, these cells were studied in isolation (monocultures) for their roles in wound repair; however, the monoculture system lacks the essential input of neighbouring cells present *in vivo*. Porous membrane inserts are now utilized to examine dual types of cells and their influence on each other. The inserts are particularly useful for studying the crosstalk between keratinocytes and fibroblasts because they mimic the physical barrier in the skin - basement membrane which separates the epidermis and dermis, and allow only the exchange of soluble factors.

The interactions between cells of the epidermis and dermis are mediated by paracrine signalling via cytokines and growth factors. Aberrant intercellular crosstalk in the skin often results in pathological conditions such scarring. Evidence has shown that hypertrophic scar-derived keratinocytes alter the phenotype of normal dermal cells and cause the development of fibrosis [17]. The state of epidermal differentiation also seems to play a role in the crosstalk. Undifferentiated keratinocytes, as opposed to differentiated keratinocytes, are able to maintain normal interactions with fibroblasts and thereby prevent dermal fibrosis [18].

On the other hand, fibroblasts express and regulate signalling factors and surface proteins in a temporospatial manner that promote keratinocyte motility and survival to activate wound re-epithelialization [19]. Without mesenchymal input, reepithelialization may be delayed and the risk of hypertrophic scar formation is increased [20]. Further, keratinocyte migration is also influenced by matrix composition [21]. Keratinocytes show better adherence and viability when seeded on a matrix containing fibroblast-released factors, and yield a better outcome in transplantation [22]. In the clinic, composite skin substitutes containing human keratinocytes and fibroblasts showed better engraftment as compared to epidermal sheet graft containing only human keratinocytes [23].

14-3-3 proteins

14-3-3 is a highly conserved small (25-30 kDa) acidic protein family, and the proteins are ubiquitously expressed in all eukaryotic cells and tissues. These proteins were first isolated from brain tissue by Moore and Pretz in 1967, and they were named based on their migration patterns on two-dimensional DEAE cellulose chromatography and starch gel electrophoresis [24]. 14-3-3 gene mutation studies elucidated the regulatory role of these proteins in development, differentiation and function of nervous system [25]. The number of detected isoforms is diverse and varies from 2 in

yeast [26, 27] to 13 in some plants [28]. Seven different 14-3-3 isoforms are isolated and characterized in mammals, and they are named, β, ε, η, γ, τ, ζ and σ, based on their elution profile on reversed phase high-performance liquid chromatography [29, 30]. In addition, α and δ are phosphorylated variants of beta and zeta, respectively. These proteins are found mainly in the cytoplasmic compartment; in addition, they can be detected in the plasma membrane and intracellular organelles such as the nucleus and the Golgi apparatus [31]. Recent studies by our group showed detection of high levels of 14-3-3 η and γ isoforms in synovial fluids from patients with joint inflammation [9].

14-3-3 proteins are known to function as intracellular adaptor or chaperone molecules which bind to many signalling proteins and regulate biological functions in cells. 14-3-3 proteins exhibit a basic monomeric structure formed by nine alpha-helices with anti-parallel distribution. These proteins form U-shaped homo- and hetero-dimers that bind to and regulate the function of more than 200 target proteins, including enzymes (serotonin N-acetyltransferase [32], tyrosine and tryptophan hydroxylase [29]), kinases (RAF1 [33], MEK kinase [34], Protein kinase C [35, 36], c-Bcr and Bcr-Abl [37], and Grb10 [38]) and phosphatases, receptors (insulin-like growth factor 1 [39], glucocorticoid receptors [40]), G-proteins, cytoskeleton proteins (vimentins [41] and keratins [42]), transporters, DNA binding proteins (histone acetyltransferase [43], TATA box binding proteins [44]), proteins involved in cell cycle control and apoptosis (cdc25 [45, 46], p27 [47], wee1 [48], BAD [49], Bax [11]), and cruciform DNA [50]. 14-3-3 binding partners are involved in the regulation of almost every cellular process including signal transduction, cell cycle control, apoptosis, transcriptional regulation, cytoskeleton rearrangement, cell adhesion, cell spreading and migration, protein localization, intracellular trafficking/targeting, protein degradation and chromosome maintenance [4, 51]. Three major mechanisms have so far been described for the regulation of 14-3-3 protein interactions with their targets. Muslin and co-workers showed that target phosphorylation on serine or threonine contained in peptide motifs of RSXpSXP or RXY/FXpSXP sequences [52], is important the for regulation of 14-3-3 binding to its targets [31]. In addition to phosphorylation of 14-3-3 targets, interactions may also depend on 14-3-3 phosphorylation as observed in the case of specific PKC isoforms [53]. It was recently found that 14-3-3 proteins are able to interact with their targets in a phosphorylation independent manner. Binding of 14-3-3 to its target may have diverse consequences including activation or inhibition of enzymatic activity, inhibition or enhancement of protein-protein interactions, subcellular localization of proteins, and promotion of post-translational modifications.

14-3-3σ and its receptor

Among 14-3-3 proteins, 14-3-3σ is a unique isoform because of its distinctive characteristics. 14-3-3σ, the expression of which is uniquely up-regulated by p53 [54] and BCRA-1 [55] in response to DNA damage, is directly involved in cell cycle regulation. In addition, this protein has a positive feedback on p53 stability and transcriptional rate [56], resulting in cell cycle arrest and subsequent DNA repair or apoptosis induction. 14-3-3σ interacts with and sequesters cyclin-dependent kinases (CDKs) in cytoplasm, leading to inhibition of G2/M progression [57-59]. Hypermethylation of CpG islands and loss of 14-3-3σ expression has been reported in various cancers including ovarian cancer [60, 61], breast cancer [62], prostate cancer [63, 64], gastric cancer [65], and hepatocellular carcinoma [66]. Several reports showed that over-expression of 14-3-3σ or reintroduction of this protein into cancer cells can inhibit uncontrolled cell proliferation and transformation in tumour cells [57]. There is a significant difference between the structure of 14-3-3σ and those of other 14-3-3 family members. This protein can only form homodimers while other isoforms form homo- and hetero-dimers. Also, there is a unique ligand binding site on the 14-3-3σ molecule formed by three amino acids Met202, Asp204 and His206 which may be responsible for its particular ligand binding properties. Finally, in contrast to other members of this protein family which appear to be ubiquitously expressed in different mammalian tissues, 14-3-3σ is specifically expressed by

epithelia cells, in particular keratinocytes [67], and its expression causes keratinocytes to exit from the stem cell state [68, 69]. Leffres *et al.* showed diffused distribution of 14-3-3σ in the keratinocyte cytoplasm, and detected a significant level of stratifin in keratinocyte conditioned medium (KCM), suggesting that this protein is also being secreted by keratinocytes [70]. Since these proteins do not have a conventional signal peptide, their externalization cannot be done through an ER/Golgi-dependent mechanism. In addition, reports have shown that their secretion is not mediated by cell lysis either [71, 72]. Chavez-Muñoz *et al.* showed that exosome-dependent secretion is the main mechanism involved in the externalization of all 14-3-3 isoforms from differentiated keratinocytes [73, 74].

The regulatory role of intracellular 14-3-3σ in cell cycle progression and cellular differentiation has been studied extensively. Biological function of the releasable form of 14-3-3σ and its involvement in the wound healing process are under active research in our lab. In 2004, Ghahary *et al.* conducted a series of experiments to identify mechanisms involved in epidermal-mesenchymal communication and wound healing process. Sequential chromatography of the active fractions of keratinocyte-conditioned medium (KCM) and peptide mapping of the candidate proteins led to identification of a keratinocyte-derived anti-fibrogenic factor known as the keratinocyte-releasable 14-3-3σ protein [70]. Extracellular 14-3-3σ function in regulation of extracellular matrix (ECM) factors and other matrix metalloproteinases

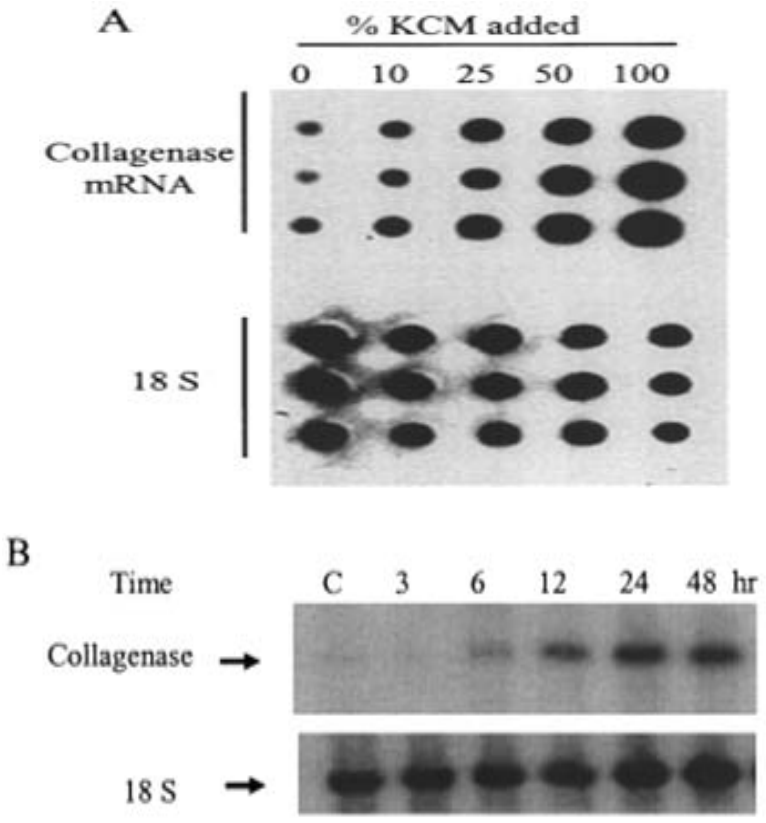

Figure 1. Dose- and time-dependent collagenase stimulatory effect of KCM on dermal fibroblasts. (**A**) Dermal fibroblasts were treated with various volumes of KCM (expressed as a percentage of total volume of KCM added) for 24 h. Total RNA was then extracted and subjected to dot blot analysis for collagenase cDNA. cDNA specific for 18S ribosomal RNA was used as a control for RNA loading. (**B**) Dermal fibroblasts were treated with KCM for 0, 3, 6, 12, 24 and 48 h. Total RNA was then extracted and subjected to Northern analysis using collagenase cDNA and 18S ribosomal RNA cDNA as the probes.

(MMPs) in dermal fibroblast were studied by using fibroblast-keratinocyte co-culture systems and treatment of fibroblasts with keratinocyte-conditioned medium or recombinant 14-3-3σ. It was shown that 14-3-3σ has a strong stimulatory effect on MMP-1 expression by dermal fibroblasts in a concentration- and time-dependent manner (Figure 1) [72]. Recent studies on the molecular mechanisms involved in the 14-3-3σ mediated stimulation of MMP-1 expression led to the identification of its receptor, CD13 or amino-peptidase N (APN) [75]. APN which belongs to the M1 family of zinc metallopeptidases is present in both soluble and membrane-bound forms. The membrane-bound form of this enzyme is involved in many cellular processes including angiogenesis, cell proliferation, differentiation, apoptosis, chemitaxis, cellular motility, invasion and adhesion, phagocytosis and antigen presentation [76]. Most of these cellular functions are highly important in the wound healing process which intrigued further research on the signalling pathways downstream of APN. Lai *et al*. [77] revealed paracrine regulation of the APN expression in dermal fibroblast by keratinocyte-derived stimuli in a dose-dependent manner (Figure 2). To study the involvement of 14-3-3σ in KCM-induced APN expression, dermal fibroblasts were treated with 14-3-3σ depleted KCM which led to the significant reduction in the APN expression. 14-3-3σ role in APN expression was further confirmed by an up-regulation of APN in fibroblasts incubated with recombinant 14-3-3σ protein. Ghaffari *et al*. [75] studies revealed that binding of the 14-3-3σ C-terminus to phosphorylated serine or threonine in APN induces p38 MAP kinase activation; in addition, they showed that 14-3-3σ mediated up-regulation of MMP-1 is eliminated by transient knockdown of APN in fibroblasts. Lam *et al*. [78] studies showed that treatment of dermal fibroblasts with 14-3-3σ leads to rapid and transient up-regulation of the principal elements of the AP-1 complex, c-jun and c-fos. AP-1 binding to the MMP-1 promoter leads to MMP-1 up-regulation. It was further shown that the expressions of collagenase-1, stromelysin-1 and -2, neutrophil collagenase, and membrane type-5 MMP in dermal fibroblasts treated with 14-3-3σ or co-cultured with keratinocytes were also increased by more than two fold [70, 79].

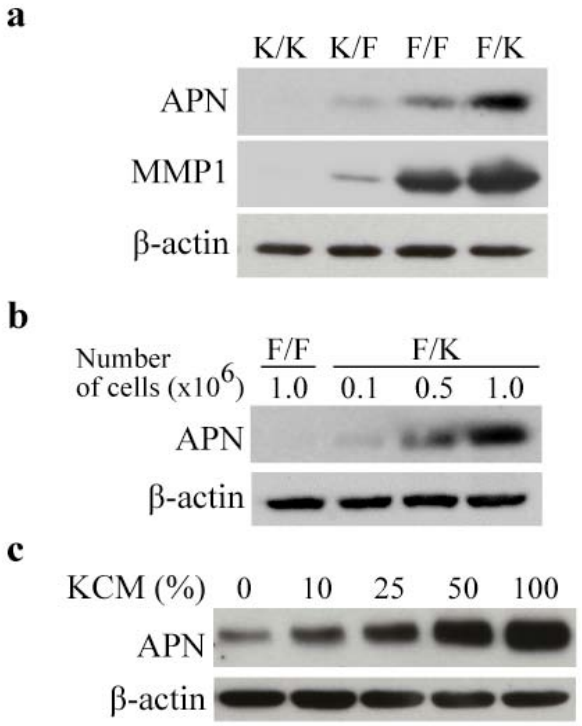

Figure 2. Dose dependent APN stimulatory effect of KCM on dermal fibroblasts. (**a**) Dermal fibroblasts were co-cultured with an equal number of keratinocytes (F/K) or fibroblasts (F/F), and vice versa (K/K and K/F). (**b**) 1.0 x 10^6 fibroblasts were co-cultured with 1.0 x 10^6 fibroblasts, or varying numbers of keratinocytes (0.1, 0.5 or 1.0 x 10^6 per well). (**c**) Different volumes of KCM were used to treat primary human fibroblasts (expressed as a percentage of total volume of KCM added). Immunoblot analysis was done to examine the APN and MMP-1 expressions in fibroblasts, and β-actin was used as a loading control.

Microarray analysis showed that the cell-surface APN receptor on fibroblasts is critical for the transmembrane signalling of certain keratinocyte-derived stimuli, including those that influence the expression of fibronectin, MMPs, and tenascin-C (unpublished data). Accordingly, dysregulation of APN may result in a fibrotic phenotype due to over-accumulation of fibronectin and reduced levels of MMPs.

Therapeutic potentials

These biochemical studies revealed a pivotal role of keratinocyte releasable 14-3-3σ and its receptor APN in the regulation of ECM remodelling by dermal fibroblasts, and suggest 14-3-3σ and APN

to be useful targets for controlling excessive wound healing in fibrotic condition [79]. In 2009, our group demonstrated the therapeutic potential of stratifin as an anti-fibrogenic/anti inflammatory factor for improving post surgical scaring. We have also designed a specific delivery system to allow in a timely manner sufficient release of an effective dose of stratifin into the wound site by embedding chitosan-conjugated stratifin embedded in PLGA and hyaluronic acid [80]. Further work in animals showed that topical administration of stratifin can significantly reduce hypertrophic scar formation in a rabbit ear fibrotic model [81].

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