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Presence of galectin-1 in the epidermal and dermal thickening of keloid tissues

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ABSTRACT

Background: Keloids are recognized as benign dermal fibroproliferative tumors that result from aberrant wound healing in susceptible individuals. Nevertheless, the etiology and pathophysiology of keloids is not fully elucidated yet. A protein that has been implicated in the wound healing process, conversion of fibroblasts into myofibroblasts and production of ECM components, inflammation, and generation of galectin-glycan complexes, is galectin-1 (Gal-1). However, its presence as well as its implications in benign dermal fibroproliferative tumors such as keloids, has not been completely elucidated. Therefore, in this study we investigated the presence and localization of Gal-1 in keloid tissues.

Methods: Seven biopsies from patients diagnosed with keloid were selected. Four normal skin specimens were obtained from patients undergoing aesthetic surgery procedures. To identification of collagen bundles, some histological sections were stained following the Mason's trichrome protocol. The presence of Gal-1 was assessed by immunofluorescence staining using confocal laser scanning microscopy.

Results: Immunostaining revealed that Gal-1 was present in the uppermost cell layers of the thickened epidermis with a gradient of expression that seems to increase from basal layer to suprabasal layers. Surprisingly, Gal-1 was particularly found outlining the cellular margin and intercellular bridges of the upper-spinous and granular keratinocytes and was absent in the epidermal keratinocytes of normal adult human skin. Interestingly, in the reticular dermis the presence of Gal-1 was noticeable in the cytoplasm of elongated fibroblasts that were forming cellular bundles of collagen and along the thick and compact collagen bundles, whereas in the dermis of normal skin, Gal-1 immunoreactivity was weak in some fibroblasts.

Conclusion: Our findings suggest that in keloid tissues Gal-1 would not only be regulating the dermal fibroblast proliferation, ECM production, particularly collagen, and abnormal vascularization, but would also be contributing to the altered stratification and terminal keratinocyte differentiation and importantly, to epidermal thickening.

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KEYWORDS

Collagen bundles; epidermal thickening; fibroblasts; galectin-1; keloid

Introduction

Keloids are recognized as benign dermal fibroproliferative tumors that result from aberrant wound healing in susceptible individuals [1-7]. They are caused by trauma, surgery procedures, burns, acne, folliculitis, vaccination, herpes zoster, infection and skin eruptions such as chicken pox [5-9]. Clinically, keloids are defined as scars that extend beyond the edges of the original wound invading adjacent healthy skin and do not regress spontaneously [1-10]. The lesions

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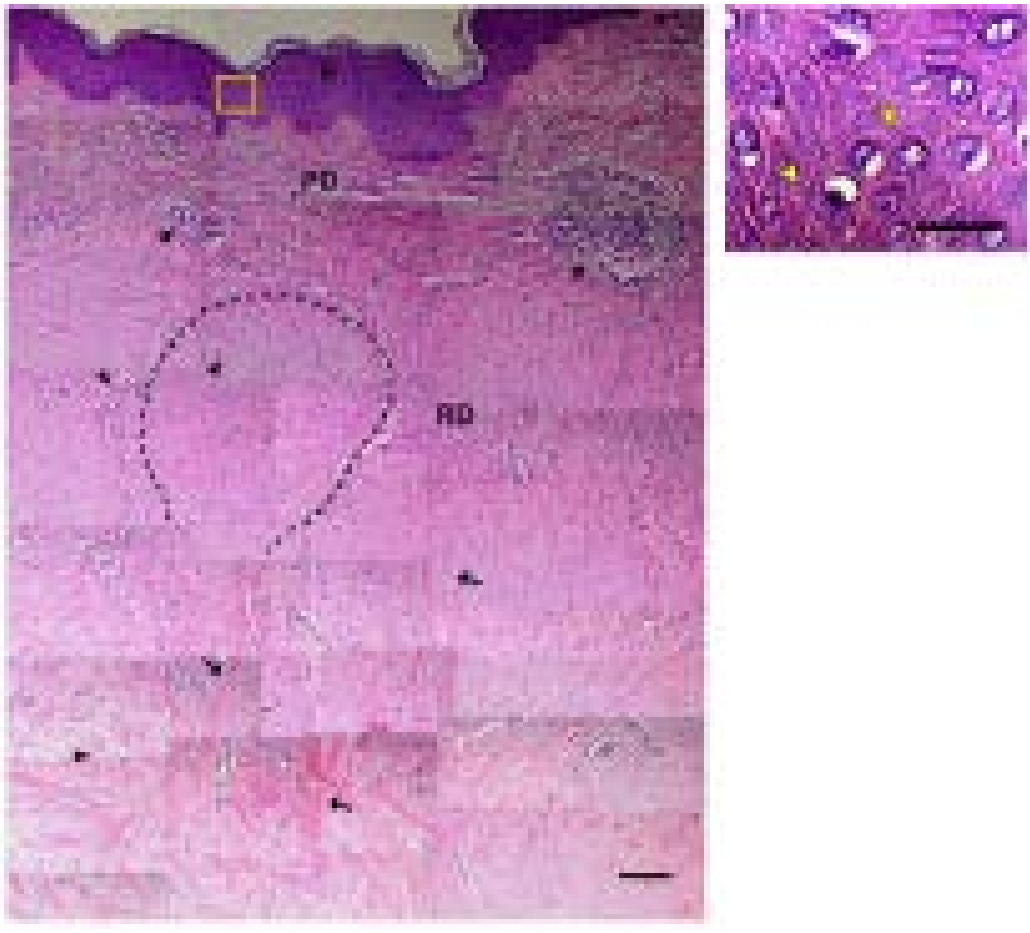

Figure 1. Hematoxylin staining (H&E) from a biopsy of keloid showing reduced rete ridges, epidermal thickening, hyperkeratosis and spongiosis. Note the presence in the dermis of thin collagen bundles parallel to the epidermis, numerous fibroblasts, microvessels (arrowheads), inflammatory cells, nodule (dashed line) containing elongated fibroblasts attached to randomly oriented collagen fibers, and thick compact collagen bundles with elongated fibroblasts attached (arrows). Enlargement shows keratinocytes organized as cohesive groups maintained together by means intercellular bridges (arrowheads). E, epidermis; PD, papillary dermis; RD, reticular dermis. Scale bar: 150 μm.

frequently occur on the anterior portion of the chest, shoulders, upper back, arms, cheeks and earlobes [1,2,5-10]. Histopathologically, keloids are defined as inflammatory disorders characterized by exhibiting a thickened dermis containing numerous fibroblasts, abnormal vascularization, increased inflammatory immune cells, abundant hyalinised collagen bundles (keloidal collagen) and extracellular matrix (ECM) components including collagen, elastin, fibronectin and proteoglycans such as syndecan and versican, mainly produced by activated fibroblasts [1-14]. These histological alterations proper of the keloid tissue have been associated with the elevated production of growth factors such as TGF-β, FGF-2, PDGF, EGF, IGF and VEGF which regulate fibroblast proliferation, ECM synthesis and angiogenesis, and pro-inflammatory cytokines including IL-1α, IL-1β, IL-6 and TNFα which promote fibroblast activation and consequently an excessive deposition of ECM components [1,2,5-7,9,10,15,16]. This is why most studies on keloids focus on dermis and few on epidermis [4,17]. However, recent studies describe the epidermis of keloids as thickened, primarily due to hyperproliferation [4,7,17] and altered terminal differentiation [18] of keratinocytes,; some of them, indicating that keratinocytes also participate in the regulation of fibroblasts activity and might contribute to keloid pathogenesis [4,7,19]. On the other hand, several studies consider genetic predisposition as an important factor in the keloid formation and progression [2,5,9,20] whilst others deem the role of local mechanical

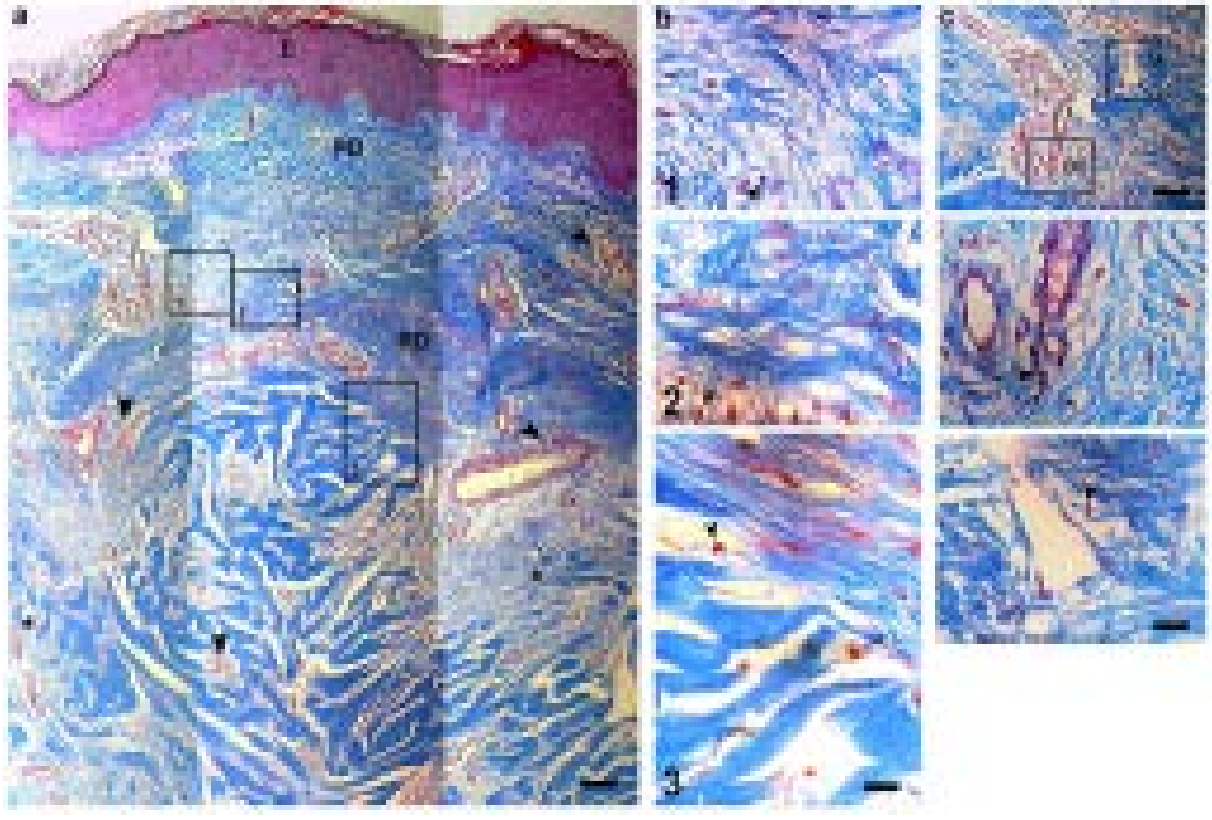

Fig 2. (A) Trichrome staining from a biopsy of keloid showing thin collagen bundles in the papillary dermis (PD) and in the upper reticular dermis (RD), and cellular and thick compact collagen bundles in the RD. Note the presence of numerous microvessels (arrowheads) and inflammatory immune cells (asterisk) localized perivascularly and dispersed between thin collagen bundles. E, epidermis. Scale bar: 100 μm. (B) Enlargements of figure (A) showing thin collagen bundles (1), cellular collagen bundles (2), and thick compact collagen bundles (3). Note the presence of inflammatory immune cells (arrowheads). Scale bar: 25 μm. (C) Detail of figure (A) showing blood (BV) and lymphatic (LV) microvessels. Scale bars: 70 μm; 25 μm.

forces as a predisposing factor of keloid formation [9]. Moreover, there is evidence suggesting that epigenetic modifications would be involved in the pathogenesis of keloids by regulation of fibroblasts activation, proliferation and apoptosis, as well as collagen and elastin synthesis [3,20-22]. Nevertheless, the etiology and pathophysiology of keloids is not fully elucidated yet [3-7,18].

A protein that has been implicated in the wound healing process [23,24], conversion of fibroblasts into myofibroblasts [24-26] and production of ECM components [26], inflammation [27], and generation of galectin-glycan complexes [28-30], is galectin-1 (Gal-1). Gal-1 is a member of the galectin family that has high affinity for N-acetyl-Lactosamine (LacNAc) disaccharides in both N- and O-glycans and is differentially expressed by various normal and pathological tissues [31]. It has been detected in the cell nucleus, cytoplasm and intracellular and extracellular sides of cell membrane, and can be secreted and found in the extracellular space [31]. Gal-1, like others galectins, participates in signaling pathways and regulates a variety of biological responses including cell growth, cell-cell and cell-matrix adhesion, cell differentiation, migration, proliferation and survival [31-34] and plays a critical role in inflammation, angiogenesis, and tumor development and progression [27,33,35,36]. In human skin, Gal-1 has been implicated in psoriasis [37,38] and malignant lesions including cutaneous cancer [39], mycosis fungoides [40,41], and melanoma [42], and scarce studies have shown that the expression of Gal-1 is upregulated in keloid tissue [43]. However, the presence, distribution, and localization of Gal-1 as well as its possible

role in benign dermal fibroproliferative tumors such as keloids, have not been completely elucidated. Therefore, we investigated whether Gal-1 is present in keloid tissues. We also sought to determine whether the presence of Gal-1 could be associated to epidermal and dermal thickening.

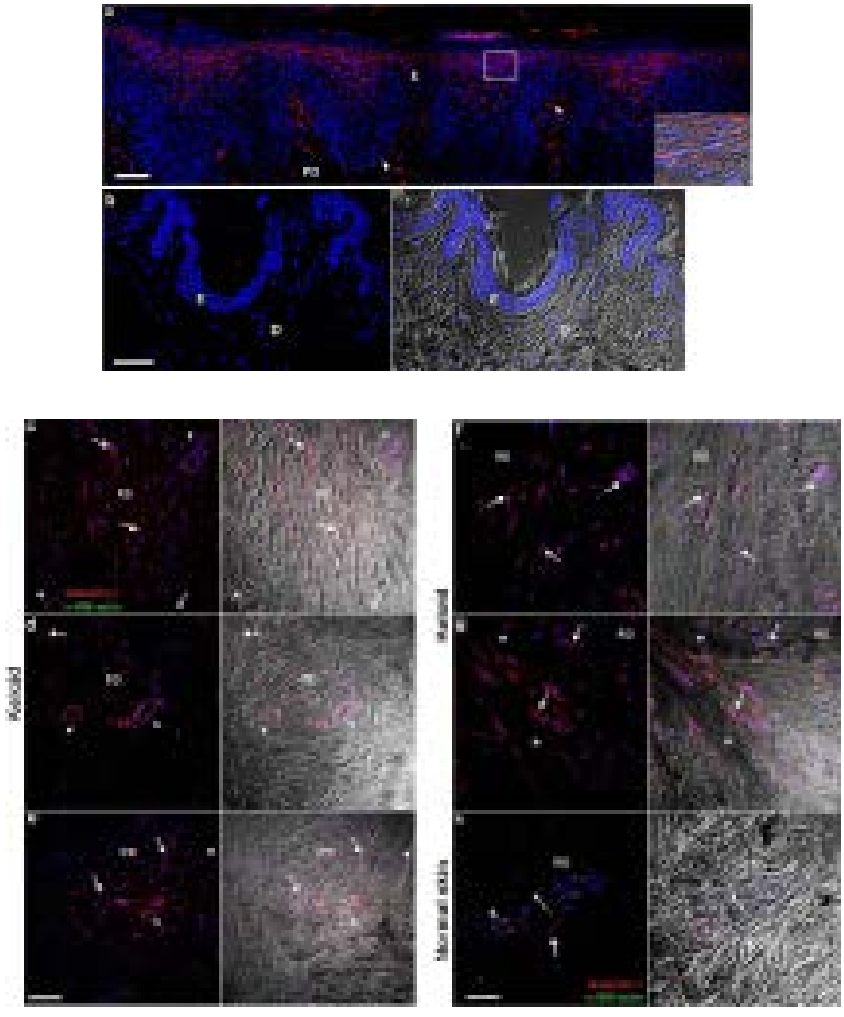

Fig 3. A series of representative CLSM fluorescence images of Gal-1 in keloid (earlobe) (A, C, D, E, F, G) and in normal skin (B, H). (A) Gal-1 is seen in the uppermost cell layers of the thickened epidermis (E) with a gradient of expression from basal to suprabasal layers. Overlay of red fluorescence and Differential Interference Contrast (DIC) images showing at high magnification Gal-1 outlining the cellular margins and intercellular bridges of the upper-spinous and granular keratinocytes in a punctate and linear array. D, dermis. Blue, DAPI staining. Scale bar: 25 μm. (B) No Gal-1 immunostaining is observed in the epidermal keratinocytes of normal adult human skin. Overlay of red fluorescence and DIC images. D, dermis. Blue, DAPI staining. Scale bar: 40 μm. (C) Double immunostaining showing the presence of Gal-1 in the cytoplasm of elongated fibroblasts (arrows) attached to thin collagen bundles and in some microvessels (arrowheads) in the upper reticular dermis (RD). Note that α-SM actin is completely absent in the fibroblasts expressing Gal-1 and those that does not it, and is present in microvessels (arrowheads). Overlay of red and green fluorescence and DIC images.Blue, DAPI staining. (D) Double immunostaining showing the presence of Gal-1 and α-SM actin in blood vessels (arrowheads) in the papillary dermis (PD). Some fibroblasts expressing Gal-1 are also observed (arrow). Overlay of red and green fluorescence and DIC images. Blue, DAPI staining. (E) Double immunostaining of a small nodule in the reticular dermis (RD). Note the presence of Gal-1 in some elongated fibroblasts (arrows) and both Gal-1 and α-SM actin in blood microvessels (arrowheads). Thick compact collagen bundles (*). Overlay of red and green fluorescence and DIC images. Blue, DAPI staining. Scale bar: 30 μm. (F) Immunolocalization of Gal-1 in cellular bundles of collagen (arrows). Blue, DAPI staining. Scale bar: 30 μm. (G) Cytoplasmic immunolocalization of Gal-1 in elongated fibroblasts attached to thick compact collagen bundles (*). RD, reticular dermis. Blue, DAPI staining. (H) Double immunostaining showing a weak Gal-1 immunoreactivity in some fibroblasts and microvessels (arrowheads) in the reticular dermis (RD) of normal skin. Note the presence of α-SM actin in the microvessels (arrowheads). Overlay of red and green fluorescence and DIC images. Blue, DAPI staining. Scale bar: 30 μm.

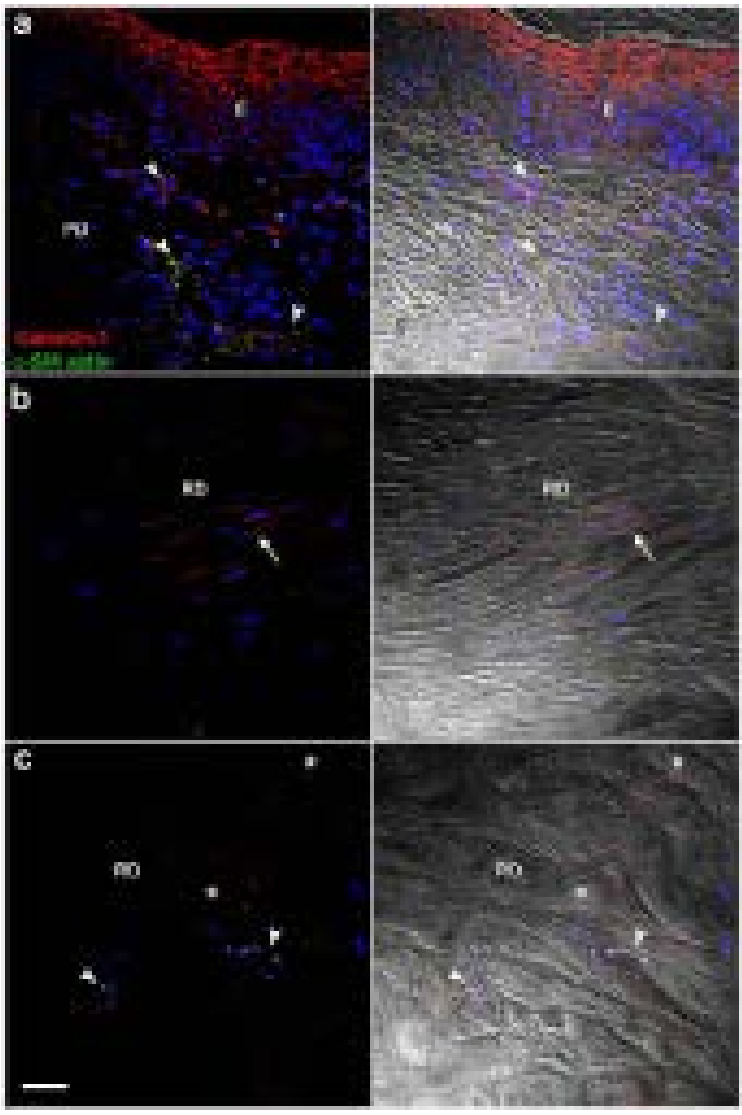

Fig 4. Double immunolocalization of Gal-1 and α-SM actin in keloid (pubis region). (A) Double immunostaining showing the presence of Gal-1 in the uppermost cell layers of the thickened epidermis (E) and both Gal-1 and α-SM actin in microvessels (arrowheads) in the papillary dermis (PD). Note that α-SM actin is completely absent in the fibroblasts expressing Gal-1 and in those that does not it. Inflammatory immune cells and fibroblasts attached to thin collagen bundles are also observed. Blue, DAPI staining. (B) Double immunostaining showing the presence of Gal-1 in elongated fibroblasts (arrow) forming cellular bundles of collagen in the reticular dermis (RD). Blue, DAPI staining. (C) Double immunostaining showing the presence of both Gal-1 and α-SM actin in microvessels (arrowheads) in the reticular dermis (RD). Thick compact collagen bundles are also observed (*). Blue, DAPI staining. Scale bar: 50 μm.

Materials and Methods

Skin Biopsies

Seven biopsies from patients diagnosed clinically and histopathologically (H&E) with keloid were retrieved from the archives of Section of Dermatopathology of SAIB. Their characteristics appear listed in Table 1. They were taken from three females and four males, ranging in age from 17 to 32 years old, with average of 23.5 years old. Four normal skin specimens were obtained from patients undergoing aesthetic surgery procedures. To identification of collagen bundles, some histological sections were stained following the Mason´s trichrome protocol. This study was approved by the Ethics Committee of the SAIB and performed according to the Declaration of Helsinki.

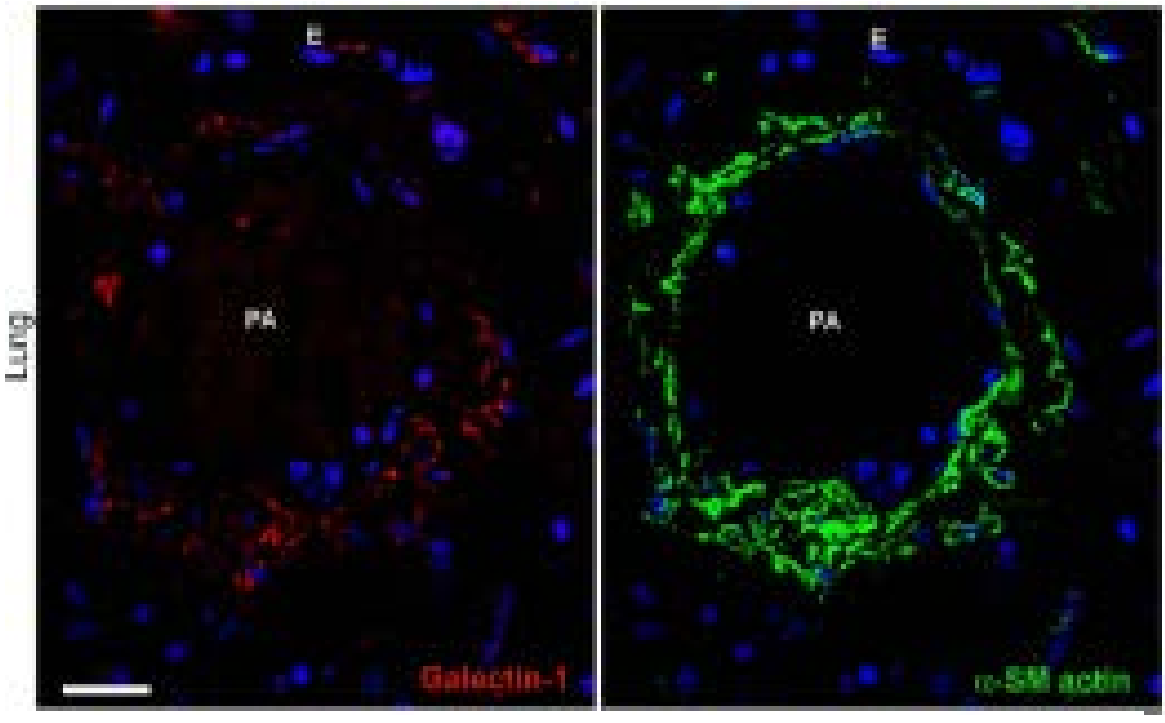

Fig 5. Double immunostaining of Gal-1 and α-SM actin in lung fibrosis. Strong immunoreactivity for Gal-1 is observed in the pulmonary artery branch (PA) and bronchiolar epithelial cells (E). Note the presence of α-SM actin in the pulmonary artery wall. Blue, DAPI staining. Scale bar: 40 μm.

Indirect immunofluorescence staining

Paraffin-embedded skin tissue blocks were cut in sections of 4 μm of thick. Paraffin sections were placed on silanized slides (DakoCytomation, Denmark), dewaxed in xylene, dehydrated through graded alcohol series (from 100 to 70%), and then placed in distilled water and equilibrated in phosphate-buffered saline (PBS) for 10 min. Non-specific antibody staining was blocked by incubating sections in PBS containing 2% bovine serum albumin (BSA) and 0.1% Tween 20 for 1 hour at room temperature (RT). The sections were incubated overnight at 4˚C in a moist chamber with rabbit polyclonal anti-human Galectin-1 antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA). Sections were washed extensively in PBS and incubated in the dark for 30 min with anti-rabbit Alexa Fluor 594 (Molecular Probes, Life Technologies, USA) at RT, followed by 15 min incubation with DAPI (4´,6-Diamidino-2-phenylindole) (Molecular Probes, Life Technologies, USA) for nuclear staining. Finally, the sections were washed in PBS and coverslipped with mounting medium (IMMU-MOUNT, Shandon, Pittsburg, PA). For double antibody staining for Gal-1 and α-smooth muscle actin (α-SM actin), the sections were incubated sequentially with the first primary antibody (anti-Gal-1) in a moist chamber overnight at 4˚C, washed in PBS and incubated for 30 min at RT with anti-mouse Alexa Fluor 592 (Molecular Probes). The sections were then washed extensively in PBS and incubated for 4 hours at RT with second primary antibody (mouse ascites monoclonal anti-α-SM actin (DakoCytomation), washed in PBS and incubated for 30 min at RT with anti-mouse Alexa Fluor 488 (Molecular Probes). Sections were washed extensively in PBS and followed by 15 min incubation with DAPI (Molecular Probes), washed in PBS and coverslipped with mounting medium (IMMU-MOUNT). A sample of lung fibrosis obtained during autopsy was used as positive control for Gal-1. All images were captured using a 1 × 81 Olympus inverted microscope with the Fluo View Confocal Laser Scanning configuration (CLSM) (Olympus America) equipped with software program FV10.ASW version 02.01.01.04 (Olympus Corporation). Image J software (NHI, Washington DC) was used for processing of contrast and brightness.

Results

Histopathological analysis of keloid tissues showed reduced rete ridges, epidermal thickening with keratinocytes organized as cohesive groups maintained together by means of intercellular bridges, hyperkeratosis, and spongiosis (Fig. 1). Important dermal modifications such as presence of thin collagen bundles parallel to the epidermis, elevated number of elongated fibroblasts, microvessels, accumulation of inflammatory cells around the microvasculature, nodules containing elongated fibroblasts attached to randomly oriented collagen fibers encompassed by thin collagen fibers and microvessels, and coexistence of thick, compact hyalinised collagen bundles with elongated fibroblasts attached, were observed (Fig. 1).

Mason's trichrome staining was also used to identify collagen bundles. Staining showed thin collagen bundles randomly organized, mainly located in the papillary dermis and in the upper reticular dermis (Fig. 2A). These thin collagen bundles seem to form cellular bundles of collagen that possibly coalesce into thick and compact collagen bundles in the reticular dermis (Fig. 2B). Staining also revealed the presence of numerous blood and lymphatic microvessels, inflammatory immune cells localized perivascularly and dispersed between thin collagen bundles, and some thin and thick collagen bundles focally fragmented distributed throughout the dermis (Fig. 2A,C).

In vivo Gal-1 immunolocalization

To investigate the presence and localization of Gal-1 in the keloid tissues, dewaxed sections were examined by immunofluorescence staining. Immunostaining analyzed by CLSM revealed that Gal-1 was present in the uppermost cell layers of the thickened epidermis with a gradient of expression that seems to increase from basal layer to suprabasal layers (Fig. 3A, 4A). Surprisingly, Gal-1 was particularly found outlining the cellular margin and intercellular bridges of the upper-spinous and granular keratinocytes in a punctate and linear array (Fig. 3A) and was absent in the epidermal keratinocytes of normal adult human skin (Fig. 3B). Immunofluorescence analysis also revealed the presence of Gal-1 in the basement membrane and in the thickened dermis of keloid tissues (Fig. 3A). In the later, this protein was detected in the cytoplasm of many elongated fibroblasts that were attached to thin collagen bundles and localized in the papillary dermis and in the nodules of the upper reticular dermis (Fig. 3C,E). Additionally, strong Gal-1 immunoreactivity was also detected in some perivascular inflammatory immune cells (Fig. 3C,D, 4A). Interestingly, in the reticular dermis the presence of Gal-1 was noticeable in the cytoplasm of elongated fibroblasts that were forming cellular bundles of hyalinised collagen and along the thick and compact hyalinised collagen bundles (Fig. 3F,G, 4B,C), whereas in the dermis of normal skin, Gal-1 immunoreactivity was weak in some fibroblasts (Fig. 3H).

There is controversy not only regarding the presence of myofibroblasts in keloid tissues [2,4,8,44] but also regarding the conversion of fibroblasts into myofibroblasts by Gal-1 [24,26,45]; therefore, we sought to determine by double-immunofluorescence staining whether α-SM actin, the most common marker for myofibroblasts identification [46], was present in the fibroblasts of keloid tissues examined. Immunolocalization demonstrated that α-SM actin was completely absent in the fibroblasts expressing Gal-1 as well as in those that did not it (Fig. 3C,D, 4A,C). Interestingly, double immunostaining also showed a strong immunoreactivity for Gal-1 and α-SM actin in both blood and lymphatic microvessels in the dermis of keloid tissues (Fig. 3C,D, 4A,C), whereas in the dermis of normal skin, moderate cytoplasmic expression of Gal-1 was observed in both blood and lymphatic microvessels and some fibroblasts (Fig. 3H). Additionally, strong immunoreactivity for Gal-1 was detected in pulmonary artery branch and bronchiolar epithelial cells when lung fibrosis was used as positive control (Fig. 5), and no immunoreactivity for Gal-1 was detected when the primary antibody was omitted or replaced with PBS (not shown).

Discussion

In the current study, we evidenced the presence of Gal-1 in the epidermal and dermal thickening of keloid tissues. Immunolocalization showed strong Gal-1 immunoreactivity in the upper-spinous and granular keratinocytes with a gradient of expression that seems to increase from the

basal layer to suprabasal layers. This particular localization and distribution of Gal-1 suggests its involvement in later stages of keratinocyte differentiation. It is important to highlight that in the keratinocytes from normal adult skin, Gal-1 was not evidenced. Remarkably, previous studies in human skin have shown that Gal-1 is not expressed by keratinocytes in normal skin [37] but can be expressed by cells in psoriasis [37,38], cutaneous malignant lesions including squamous cell carcinoma [39], mycosis fungoides [40,41], and melanoma [42], while in keloid tissues, proteome analysis of keloid proteins performed by Ong et al. revealed that the expression of Gal-1 is upregulated compared to normal skin [43]. However, the role of Gal-1 in skin lesions is still unclear. In this respect, recent in vitro studies have shown that Gal-1 and Gal-3 secreted by malignant T cells regulates proliferation of keratinocytes and induce disorganization of the outer layers of the epidermis, accompanied by decreased expression of the terminal differentiation marker involucrin [47]. Similarly, studies related to galectins expression and function have shown a differential pattern of Gal-1 and Gal-3 expression during the course of wound healing with Gal-1 being upregulated at the early stages of healing and Gal-3 at the later stages [23,48,49]. Of particular interest, is that increased epidermal thickness not attributable to hyperproliferation, but possibly caused by abnormal terminal differentiation of keratinocytes that involves an increased expression of involucrin in the spinous and granular layers, has been recently reported in keloid scars [18]. Regarding the expression of Gal-1 in normal and pathological tissues, some studies highlighted that Gal-1 is detected in the cell nucleus, cytoplasm and intracellular and extracellular sides of cell membrane regulating cell growth, cell-cell and cell-matrix adhesion, cell proliferation, differentiation, migration and survival [31-34], and that its expression might be induced by elevated levels of pro-inflammatory cytokines and growth factors [27,33,50,51]. Therefore, it is possible that Gal-1 contributes to the abnormal stratification and differentiation of keratinocytes in keloid tissues by upregulation of involucrin expression, and that a differential regulation of Gal-1 and Gal-3 expression would be occurring during the progression of keloid considering that Gal-1 was reduced or absent in normal epidermis and was present in the epidermal thickening of keloids, while Gal-3 appears gradually in normal epidermis and absent or decreased in the epidermal thickening of some skin lesions such as psoriasis, basal cellular carcinoma and lichen planus [37,52,53]. Also, it is possible that some pro-inflammatory cytokines and growth factors released from keratinocytes and/or immune cells could be inducing Gal-1 expression.

In this study, immunofluorescence also evidenced an augmented intracellular immunoreactivity for Gal-1 in many elongated fibroblasts and few immune cells in the dermal thickening of keloids. We suggest that this increased immunoreactivity for Gal-1 in the fibroblasts could be induced by certain mediators that are released from inflammatory and endothelial cells at the inflammation sites, and that Gal-1 in turn, would induce fibroblasts proliferation and ECM production considering that keloids are defined as chronic inflammatory disease characterized by the presence of numerous fibroblasts, abnormal vascularization and increased inflammatory immune cells [54]. In support of this, studies in animal models of fibrosis have provided evidence that Gal-1 stimulates the proliferation and collagen production of hepatic and pancreatic stellate cells with the possible participation of inflammatory immune cells [55-57]. Similarly, recent studies related to Gal-1 expression have shown upregulated levels of Gal-1 and collagen I in lung fibrosis [51]. Nonetheless, we can not exclude the possibility that in keloid tissues Gal-1 might also regulates the dermis inflammation and fibroblasts proliferation; moreover, considering that intracellularly Gal-1 inhibits both the soluble and cellular mediators of the inflammatory response in acute and chronic inflammation [27], and regulates the liver inflammation and hepatic cells proliferation in liver regeneration [58]. Of note, a protective role for Gal-1 during acute myocardial infarction by preventing cardiac inflammation has been proposed [59].

In this study, we also evidenced the presence of numerous blood and lymphatic microvessels exhibiting an increased immunoreactivity for Gal-1 in the dermal thickening of keloids. Interestingly, abnormal vascularization has been associated not only with pronounced proliferation

of endothelial cells [60] and hypoxia [11,61], but also with an elevated Gal-1 expression [62,63]. In fact, studies have shown that Gal-1 can induce endothelial cell proliferation [64,65] and that Gal-1 expression can be regulated by hypoxia [66,67]. Also of significance, some studies have provided evidence that local hypoxia not only promotes keratinocyte motility and migration during wound healing [68] but also stimulates the deposition of collagen by fibroblasts in wounds and keloids [69-71] and abnormal vascularization in keloids [11]. Thus, we believe that the increased immunopositivity for Gal-1 observed in dermal thickening would be associated with the fibroblasts proliferation, excessive deposition of collagen and abnormal vascularization; moreover, considering that hypoxia seems to be an important element in the pathogenesis of keloids [4,70].

There is controversy not only with respect to the presence of myofibroblasts in keloid tissues [2,4,8,44] but also over the conversion in vivo of dermal fibroblasts into myofibroblasts in wound healing induced by Gal-1 [24,26,45]. In fact, different studies in vivo have reported that myofibroblasts are the predominant cell type present in keloid tissues [4,44] while others report that they are absent [2,8] and that in animal models Gal-1 induces the conversion of dermal fibroblasts into myofibroblasts during wound healing [23,45]. Consequently, we also examined whether α-SM actin was present in the fibroblasts expressing Gal-1. Immunolocalization demonstrated that α-SM actin was absent in the dermal fibroblasts in spite of fact that many of them expressed Gal-1. Thus, these observations confirm that in keloid tissues myofibroblasts are absent and suggest that in vivo the expression of Gal-1 in keloid fibroblasts is not associated with the possible conversion of fibroblasts into myofibroblasts.

Taken together, our findings suggest that in keloid tissues Gal-1 would not only be regulating the dermal fibroblast proliferation, ECM production, particularly collagen, and abnormal vascularization, but would also be contributing to the altered stratification and terminal keratinocyte differentiation and importantly, to epidermal thickening.

However, additional studies with a larger sample of keloid tissues will be necessary not only to establish the role of Gal-1 but also to gain new insights into the underlying mechanisms that regulate its expression and functioning.

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Disclosure

The authors disclose that they have no conflict of interest.

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