

Wound Healing Effects of a Lipocalin-Derived Peptide

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Abstract

The role of lipocalins in wound healing is currently unknown, although the involvement of these proteins in injury response has been reported. This study aimed to investigate the effects of a peptide comprising the sequence of a previously described lipocalin conserved motif (pM2b), in an experimental model of skin lesion. Circular full-thickness wounds in the skin of rat dorsum were treated with pM2b or saline as control, and allowed to heal, keeping the wound occluded and moist. During wound healing, the following parameters were evaluated in the regenerating tissue: wound closure, collagen content, glycosaminoglycans (GAGs) and metalloproteinase (MMP) activity. In addition, tissue sections were subjected to histological analysis. Treatment with pM2b promoted an overall improvement in wound healing and tissue repair, with distinctly marked signals in the early stages of wound healing, such as the presence of histiocytes, fibroblasts and thick collagen bundles, as well as early reepithelization in the recovering tissue. In the latter stages, pM2b-treated wounds showed a better resolution of wound healing, with evidence of regeneration and reduced scars. The regenerating tissue showed collagen increase, no significant changes in the total amount of GAGs, and increased MMP-2 activity, in comparison with control lesions. The results suggest that lipocalins which share the sequence motif related to pM2b can play a role in wound healing. The lipocalin-derived peptide can serve as a tool to develop new pharmaceuticals and formulations to aid wound healing.

Keywords Lipocalin; Lopap; Amino acid motif; Signature sequence; Peptide; Wound healing

Introduction

A diversity of biological molecules is involved in triggering and regulation of wound healing and tissue repair processes. Interaction between extracellular matrix (ECM) and cells, as well as modulation of cell responses play important roles in the dynamic regulation of wound healing and establishment of normal tissue morphology and function [1-3]. Among the decisive events distinguished in wound healing are the primary hemostasis, wound contraction, cell recruitment, orchestrating of cell responses by growth factors and cytokines, and the synthesis of ECM components [4].

Collagen is the main ECM component, important to maintaining tissue integrity. During tissue repair, fibroblasts and myofibroblasts are recruited to the new forming tissue and synthesize collagen. Rapid collagen synthesis and arrangement in a well-organized pattern, equilibrated with tissue remodeling that involves the activation of matrix metalloproteinase are critical factors, which determine the success of tissue repair, and scar prevention [5,6].

Recently, lipocalins have arisen in literature as players in injury response and morphogenic processes [7-12]. Lipocalins are multifunctional proteins that can modulate cell survival, differentiation and immune responses [13-15]. We have previously demonstrated that a signature sequence is involved in the modulation of cell responses by lipocalins and have described a peptide (pM2b), which comprises the conserved sequence motif related to the lipocalin

signature sequence [16,17]. In vitro assays showed this lipocalin-derived peptide triggers anti-apoptotic activity in endothelial cells and neutrophils, and is able to modulate fibroblast responses, inducing the synthesis of ECM proteins, and expression of inflammatory and cell survival mediators [16,18]. In vivo assays, pM2b also induced increased collagen synthesis in normal dermis [18]. In the present study, we evaluated the healing effects of pM2b in skin full-thickness wound.

Materials and Methods

Peptide and reagents

The synthetic peptide (pM2b) with the amino acid sequence YAIGYSCKDYK-OH was obtained from Orpegen Pharma (Heidelberg, Germany). All other reagents were of analytical grade.

Full-thickness skin lesion model

Male Wistar rats aged 6-8 weeks and weighing 120-150 g were obtained from the Central Animal Breeding House, Butantan Institute. The animals were fed with standard pellet diet and water ad libitum. All procedures were approved by the institutional animal care and use committee. Rats were anesthetized with a mixture of ketamine (75 mg.kg⁻¹ im) and xylazine (10 mg.kg⁻¹ im). The dorsum was shaved and disinfected with ethanol. Six full-thickness round sections (three wounds on each side) of 6 mm diameter were aseptically made on the skin using a metallic punch. The wounds on the right side were topically treated with a single dose of the peptide (50 µl, 230 nM

pM2b) while the wounds on the left side were treated with the vehicle (saline) as control. Each wound was dressed with Bioclusive transparent dressing (Johnson & Johnson, Arlington, TX, USA). Full-thickness excisions were taken as a reference of normal intact skin, considered as control of intact dermis. At intervals of 3, 7, 14, and 21 days after wounding rats (n=8) were euthanized, and full-thickness skin samples from the healing wounds were excised for posterior analyses. Before excision, the size of each lesion was measured and the wounds were examined to evaluate wound contraction and the healing tissue. The wound area was measured using the formula: wound area (mm²) = (a x b) x $\pi/4$, where a and b are the major and minor axes (mm diameter) of the wounds, respectively [19]. Data are presented as percentage of the initial wound area.

Histological analysis

Skin samples collected at the time of wounding and at all-time intervals after wounding were immediately fixed in a solution of 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 3 μ m, and stained with hematoxylin-eosin (HE) to analyze tissue morphology, and picrosirius red for collagen analysis. Slides were examined under light microscopy. Images were acquired at 250x magnification. Peptide-treated lesions were compared with saline-treated lesions at each time intervals.

Collagen analysis

Collagen analysis was done as previously described [20]. Skin samples collected at the intervals of 7, 14, and 21 days after wounding were incubated for 4 h at room temperature with pepsin in 0.5 M acetic acid, pH 2.5, and then maintained at 4°C during 20 h. Then, the samples were centrifuged at 8 000 g for 30 min. The supernatants were lyophilized and dissolved with 0.5 M acetic acid. The total amount of protein was measured by the method of Bradford, using a standard curve of bovine serum albumin [21].

Analysis of glycosaminoglycans

Glycosaminoglycans (GAGs) were extracted as previously described [22]. Skin samples collected at the intervals of 7, 14, and 21 days after wounding were incubated for 24 h in acetone and delipidated in methanol: chloroform (1:2). Dry tissues were rehydrated in 100 mM sodium acetate, pH 5.0, with 5 mM cysteine and 5 mM EDTA and incubated with 20 mg/mL papain for 24 h at 60°C. After 20 min centrifugation at 3000 g, supernatants were collected and GAGs were precipitated by addition of 0.5% cetylpyridinium chloride (CPC). The CPC-GAG complex was dissociated with 2 M sodium chloride with ethanol (100:15). GAGs were washed in ethanol and quantified by the carbazole method, using a galacturonic acid standard curve [23].

Matrix metalloproteinase activity assay

Matrix metalloproteinases (MMPs) were extracted from the recovering tissue collected at all-time intervals and analyzed by gelatin zymography, as previously described [24]. The samples were homogenized (1/10, w/v) in 100 mM Tris-HCl, pH 7.6, containing 200 mM NaCl, 100 mM CaCl₂ and 1 % Triton X-100, at 4°C for 24 h. After centrifugation at 12000 g for 15 min, the supernatants were collected and the protein concentration was measured by the method of Bradford, using a standard curve of bovine serum albumin [21]. The samples were subjected to electrophoresis at 100 V, 4°C, using 7.5% polyacrylamide gels containing 1 mg/mL co-polymerized gelatin. The

MMP activity was visualized as clear bands against a dark-blue background.

Statistical analysis

Statistical analysis was performed using Student's t-test. P values less than 0.05 were regarded as statistically significant.

Results

Wound closure and macroscopic changes

A slight improvement in wound closure was observed in the peptide-treated lesions, with significant differences at the third and fourteenth day after treatment (Figure 1).

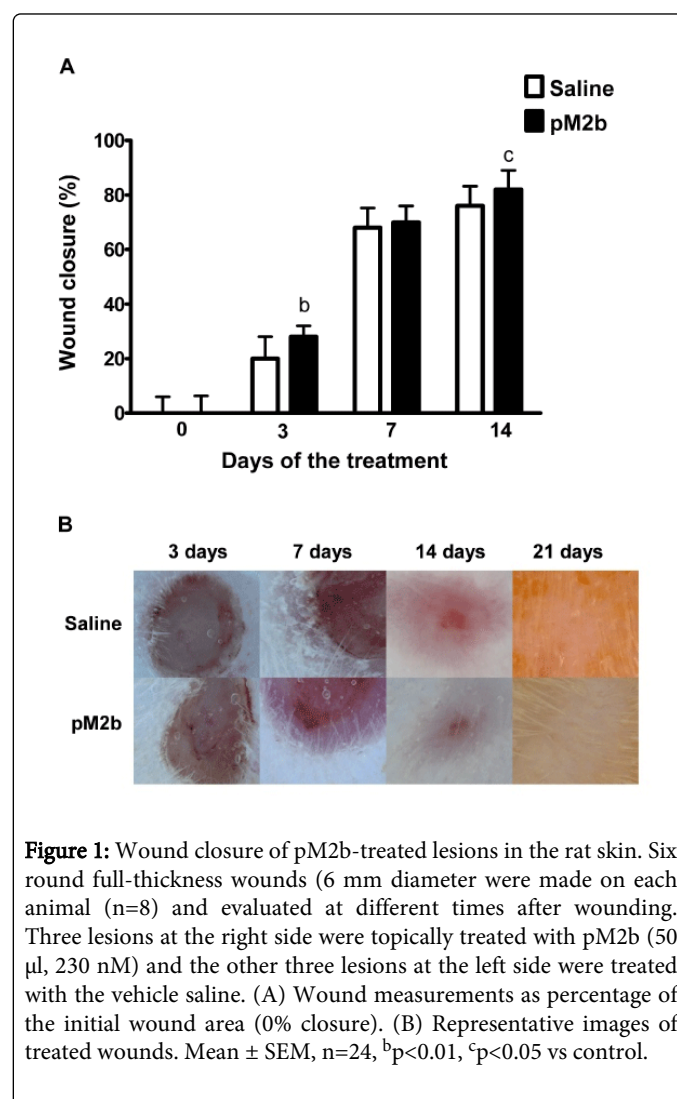


Figure 1: Wound closure of pM2b-treated lesions in the rat skin. Six round full-thickness wounds (6 mm diameter) were made on each animal (n=8) and evaluated at different times after wounding. Three lesions at the right side were topically treated with pM2b (50 μ l, 230 nM) and the other three lesions at the left side were treated with the vehicle saline. (A) Wound measurements as percentage of the initial wound area (0% closure). (B) Representative images of treated wounds. Mean \pm SEM, n=24, ^bp<0.01, ^cp<0.05 vs control.

Dermoscopy is a non-invasive diagnostic tool that allows the recognition of morphologic structures not visible skin. Were analyzed and highlighted the following structures in the skin include hair shafts, hair follicle openings, the peri-follicular epidermis and cutaneous microvasculature. After three days, the control group lesions presented bleeding, discrete epithelial disarrangement and edematous areas in the wound edges. On the other hand, pM2b-treated lesions showed

only discrete bleeding points and markedly retracted edges (Figure 1). At the seventh day, control lesions showed few retracted edges, intense bleeding points, vascular congestion and discrete necrosis segments, while peptide-treated lesions showed reepithelization and markedly retracted edges. Fourteen days after wounding, control lesions had accentuated vascularization with partial contraction and pM2b-treated lesions showed wound contraction and poor vascularization when compared with control. After twenty one days, peptide-treated lesions showed repaired tissue of normal appearance and absence of scars. Hypertrophic scars were accounted in approximately 30% of the wounds treated with saline.

Histological aspects of HE stained sections

The dermis control rats were composed by dense connective tissue, intermingled by skin appendages. Deep aspects of the specimens show striated muscle bundles and adipose tissue (Figure 2).

(Group saline - initial) ulcerated fragment of skin covered with a leuco-fibrinouspseudo membrane. Acute and chronic inflammatory infiltrate and edema, extending to the deep aspects of the dermis are also observed; (Group treated pM2b - 0 Day) ulcerated fragment of skin covered with a leuco-fibrinouspseudo membrane, present inflammatory infiltrate, with presence of histiocytes and edema.

(Group saline - 7Days) ulcerated fragment of skin covered with a leuco-fibrinouspseudo membrane over well-organized granulation tissue. Note the perpendicular distribution of blood vessels; (Group pM2b - 7Days) Well-organized granulation tissue under area of early reepithelization. Note the presence of fibroblasts and the perpendicular distribution of blood vessels.

(Group saline - 14Days) Fragment of skin with evidence of early reepithelization. In the dermis, remnants of granulation tissue, mild inflammatory infiltrate and hemorrhagic exudate are observed. Collagen fibres are organized in parallel bundles; (Group pM2b - 14Days) Fragment of skin with evidence of intenseregeneration. In the dermis, mild, mild inflammatory infiltrate and small hemorrhagic foci are observed. Collagen fibers are organized in parallel bundles.

(Group saline - 21Days) Fragment of re-epithelized skin with areas ortho keratinized epidermis. Dermis is composed by dense connective tissue with parallel thick collagen bindles intermingled with small blood vessels, mild inflammatory infiltrate and edema. (Group pM2b - 21Days) Fragment of re-epithelized skin with extensive ortho keratinized epidermis. Dermis is composed by dense connective tissue with parallel thick collagen bindles intermingled with small blood vessels, absence inflammatory infiltrate and edema.

The HE-stained slides of samples from saline-treated lesions, obtained three days after wounding, showed ulcerated fragment of skin covered with a leuco-fibrinouspseudo membrane. Acute and chronic inflammatory infiltrate and edema, extending to the deep aspects of the dermis were also observed. Samples from pM2b-treated wounds showed intense ulcerated fragment of skin covered with a leuco-fibrinouspseudo membrane. Acute and chronic inflammatory infiltrate, with presence of histiocytes and edema, extending to the deep aspects of the dermis were also observed (Figure 2).

After seven days, ulcerated fragment of skin covered with a leuco-fibrinouspseudo membrane over well-organized granulation tissue was observed in control samples. Note the perpendicular distribution of blood vessels. The pM2b peptide-treated lesions showed well-organized granulation tissue under area of early reepithelization. Note

the presence of proliferation fibroblasts and the perpendicular distribution of blood vessels (Figure 2).

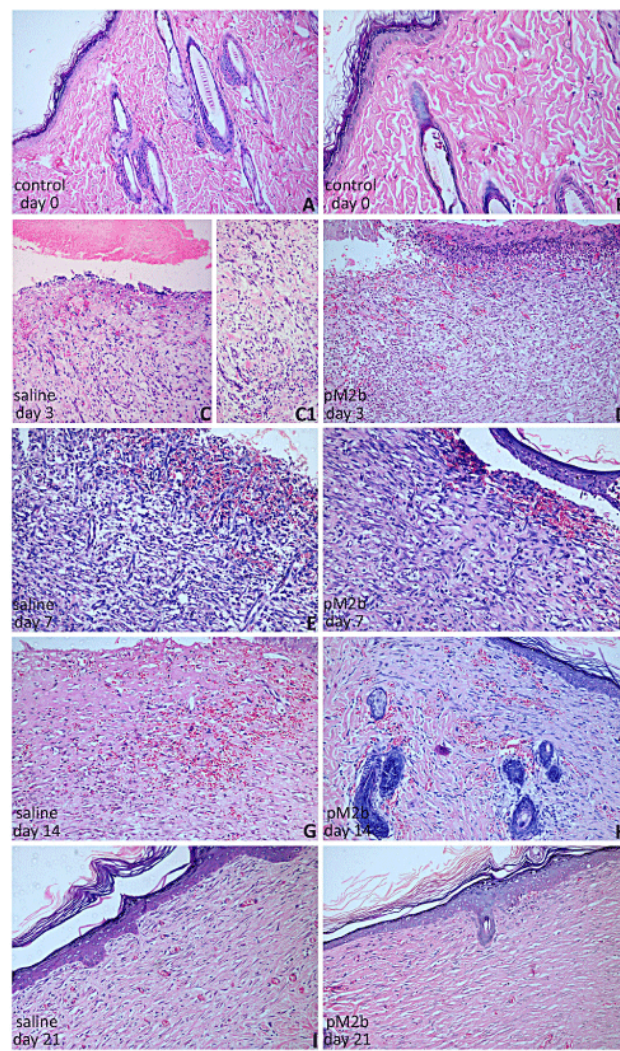


Figure 2: HE stained sections of post-wounding recovering tissue. Photomicrographs of mice skin and scar tissue with or without treatment, stained with HE technique. Full-thickness excisions from the wounding site topically treated with pM2b (50 μ l, 230 nM) or saline were subjected to histological analysis. Fragment of control skin with ortho keratinized epidermis. Dermis is composed by dense connective tissue, intermingled by skin appendages. Deep aspects of the specimens show striated muscle bundles and adipose tissue.

At the fourteenth day, control samples showed fragment of skin with evidence of discrete reepithelization cutaneous process. In the dermis, remnants of granulation tissue, mild inflammatory infiltrate and hemorrhagic exudate were observed. Collagen fibers were organized in parallel bundles. Samples from pM2b-treated lesions showed fragment of skin with evidence of active regeneration. In the dermis, mild inflammatory infiltrate and rare hemorrhagic foci were observed. Collagen fibers were organized in parallel bundles (Figure 2).

Twenty one days after wounding, both control and peptide-treated samples presented fragment of reepithelized skin with ortho keratinized epidermis. In groups peptide treated, dermis was composed by dense connective tissue with parallel thick collagen bundles intermingled with small blood vessels, absence inflammatory infiltrate and edema (Figure 2).

Histological aspects of picosirius-stained sections

Picosirius-stained slides of recovering tissue from group control lesions three days after wounding had thin collagen bundles (stained red), separated by edema extending to the entire dermis. Lesions treated with pM2b showed thin collagen bundles, separated by edema extending to the superficial portion of the dermis (Figure 3).

(Group saline - initial): thin collagen bundles (stained red), separated by edema extending to the entire dermis; (Group saline - 3Days) thin collagen bundles (stained red), separated by edema extending to the superficial portion of the dermis.

(Group pM2b - 3Days) thin collagen bundles (stained red), organized in a parallel architecture. Compare with the normal thick collagen bundles (stained bright red) in the adjacent area.

(Group pM2b - 7Days) thin collagen bundles (stained red), organized in a parallel architecture. In some areas of the surgical defect, nodules of more mature collagen (bright red) can be observed. Compare with the normal thick collagen bundles (stained bright red) in the adjacent area.

(Group pM2b - 14Days) thin collagen bundles (stained red), organized in a parallel architecture, intermingled by mild edema. Compare with the normal thick collagen bundles (stained bright red) in the adjacent area.

(Group saline - 21Days) dermis showing thick bundles of collagen, stained in red, intermingled by mild edema; (Group pM2b 21Days) dermis showing thick bundles mature of collagen, intermingled by mild edema.

At the seventh day, thin collagen bundles, organized in a parallel architecture were observed in control. Compare with the normal thick collagen bundles in the adjacent area. Samples from peptide-treated lesions presented thin collagen bundles, organized in a parallel architecture. In some areas of the surgical process, nodules of more mature collagen (bright red) were observed. Compare with the normal thick collagen bundles in the adjacent area (Figure 3).

At the fourteenth day, control samples presented thin collagen bundles, organized in a parallel architecture, intermingled by mild edema. Compare with the normal thick collagen bundles in the adjacent area. On the other hand, pM2b-treated samples showed thick collagen bundles, organized in a parallel architecture, intermingled by mild edema (Figure 3).

Twenty one days after wounding, the recovering tissue from control and pM2b-treated wounds presented dermis showing thick bundles of collagen, stained in red, intermingled by mild edema (Figure 3). Compare with normal dermis prior to wounding showing thick bundles of collagen (Figure 3).

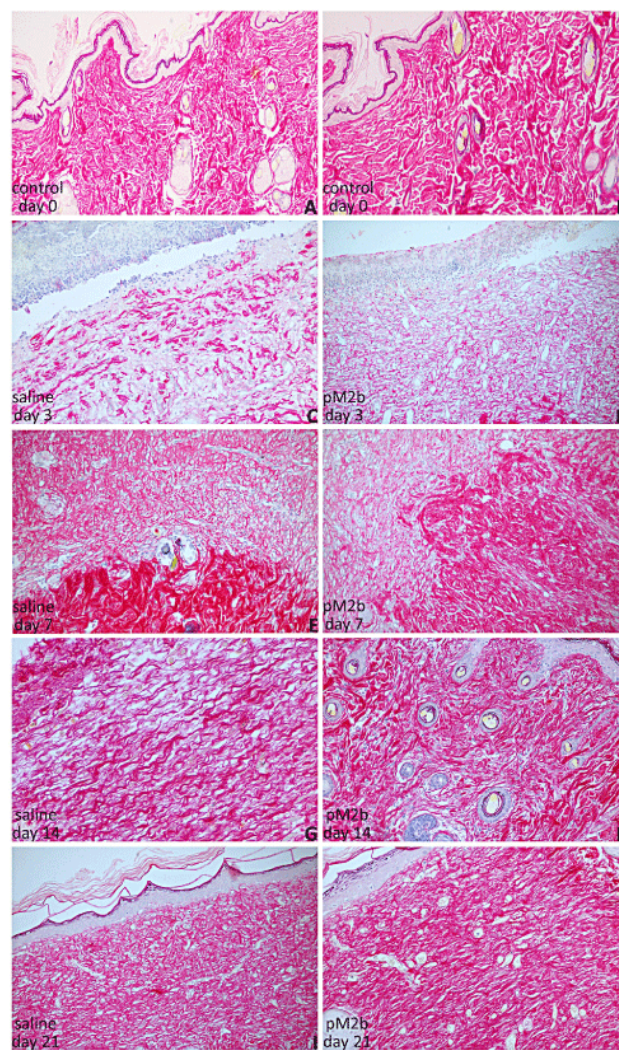


Figure 3: Picosirius stained sections of post-wounding recovering tissue. Full-thickness excisions from the wounding site topically treated with pM2b (50 μ l, 230 nM) or saline were subjected to histological analysis. Slides containing mice skin were stained with picosirius red, and examined by bright field. The samples dermis group controls showing thick bundles of collagen, stained in red.

Extracellular matrix components

Analysis of protein extracts showed a significant increase of collagen content in pM2b-treated wounds, seven and fourteen days after wounding (Figure 4).

The total amount of GAGs measured were $5.33 \pm 2.8 \mu\text{g/mL}$ and $5.99 \pm 2.5 \mu\text{g/mL}$ seven days after wounding, in control and pM2b-treated samples, respectively. After fourteen days, samples from saline-treated wounds showed $5.86 \pm 1.81 \mu\text{g/mL}$ and samples from pM2b-treated wounds $6.25 \pm 2.0 \mu\text{g/mL}$. After twenty one days, measures of $1.79 \pm 0.59 \mu\text{g/mL}$ and $1.84 \pm 0.55 \mu\text{g/mL}$ were observed in control and pM2b samples, respectively. Despite a slight increase in the total

amount of GAGs were observed in the samples from peptide-treated wounds, there were no significant differences.

A significant increase of protein concentration was observed in the MMP extracts from pM2b-treated lesions, three days after wounding (Figure 5).

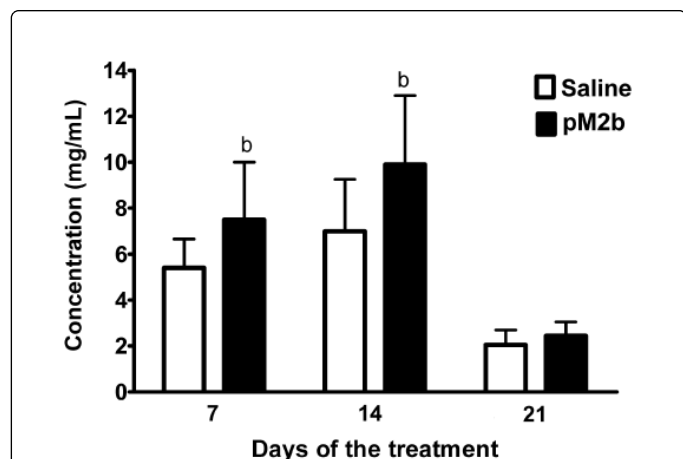


Figure 4: Effect of pM2b on collagen content in the healing tissue. Protein measurements in collagen extracts of tissue samples from the wounds topically treated with pM2b (50 μ l, 230 nM) or saline, measured by Bradford assay. Mean \pm SEM, n=8, ^bp<0.01 vs control.

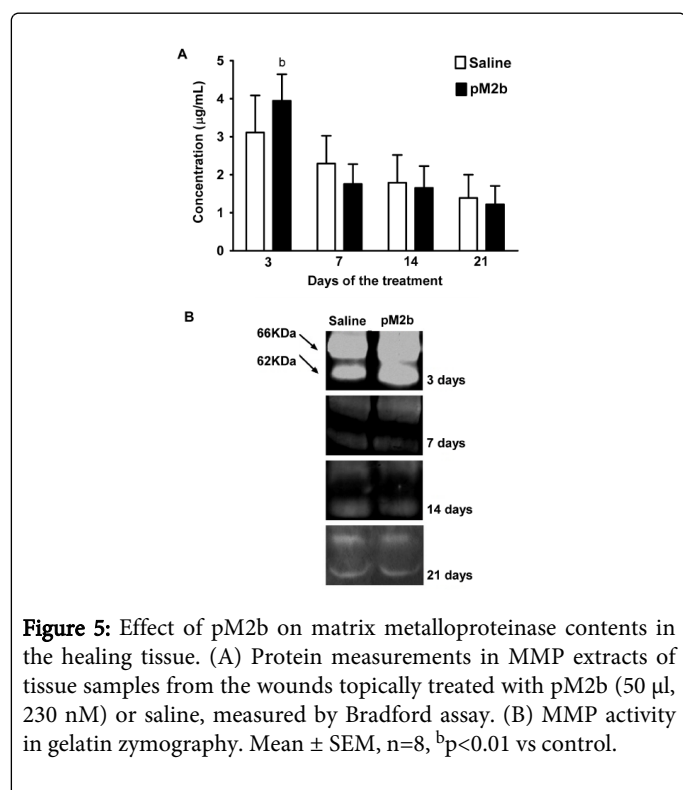


Figure 5: Effect of pM2b on matrix metalloproteinase contents in the healing tissue. (A) Protein measurements in MMP extracts of tissue samples from the wounds topically treated with pM2b (50 μ l, 230 nM) or saline, measured by Bradford assay. (B) MMP activity in gelatin zymography. Mean \pm SEM, n=8, ^bp<0.01 vs control.

In addition, the zymography analysis showed an increase in proteolytic activity of extracts from the peptide-treated wounds (Figure 5B), with higher intense bands, in the samples collected at the third day after wounding. Two clear bands of approximately 66 and 62

kDa were observed, which corresponds to the molecular mass of the intermediate and active forms of MMP-2, respectively.

Discussion

Healing is the interaction of a complex cascade of cellular events that generates resurfacing, reconstitution, and restoration of the tensile strength of injured skin. Wound healing occurs in overlapping steps recognized as hemostasis, inflammation, proliferation and remodeling, which involves interactions between cells and biochemical mediators around the injury site [2,4]. Growth factors and cytokines are the main described endogenous factors mediating such events [25]. However, the lipocalin roles in these processes are not known so far. The present study was carried out to investigate the possible beneficial effects of a lipocalin-derived peptide (pM2b) in wound healing following a lesion-induced model in rats. The peptide was designed based on the amino acid sequence of Lopap (Lonomiaobliqua prothrombin activator protease), a lepidop teranlipocalin [26] previously reported that displays anti-apoptotic activity, also inducing a set of cell responses, which involves increased expression of cell adhesion molecules, nitric oxide, PGI2 and IL-8, in addition to leukocyte recruitment [27-29].

This peptide reproduced the anti-apoptotic activity of Lopap and other lipocalins, such as purpurin and prostaglandin D synthase [16]. In addition, pM2b was able to induce the synthesis of extracellular matrix proteins in fibroblast culture and modulate pro-inflammatory cytokine signaling [18]. pM2b is related to a conserved lipocalin sequence motif, which has been proposed as a signature sequence with roles in cell modulation [16,17].

This study corroborates with the previous findings, indicating that pM2b displays modulation effects in wound healing in vivo. Macroscopic observations of the recovering wounds are in accordance with the results of histological analysis. The cell modulation activity displayed by pM2b might be involved in the chronic inflammatory infiltrate observed and the migration of histiocytes and fibroblasts to the site of injury.

The modulation effect of pM2b in fibroblasts inducing cytokine signaling, such as IL1- β , IL-6R, CXCR-1 and CXCR-2 [16], could contribute to the beneficial effect of peptide treatment in wound healing herein observed. Indeed, a decrease of these mediators has been associated with delayed wound healing [30,31].

Activated fibroblasts present in the recovering wounds that were treated with the peptide can be responsible for the increase in collagen production and organization fibrillar. Collagen is the most abundant component of extracellular matrix synthesized by fibroblasts. During wound healing, collagen deposits provide a matrix for anchorage and migration of cells and generate tension forces [2].

Other effect observed in pM2b-treated wounds was the increased levels of MMPs, especially active MMP2. The increase of MMP2 activity observed in pM2b-treated wounds, in the early stage of wound healing, may be associated with cell migration and reepithelization. MMPs play important roles in wound healing, regulating tissue remodeling, ECM composition, cell migration, and the function of non-ECM molecules, such as growth factors and their receptors, cytokines and chemokines, and adhesion receptors [32]. MMP2 is expressed by keratinocytes and fibroblasts and its level is increased by the interaction between these cells during wound healing [33].

Despite the involvement of lipocalins in tissue regeneration and developmental process has been widely suggested, this is the first

description of a lipocalin-derived molecule being evaluated for its potential effects in wound healing. Further studies should focus on the investigation of signaling pathways involved in tissue responses to pM2b treatment, the optimized dosage, absorption and half-life. Results altogether suggest that lipocalins, through a conserved motif, can play a role in healing and tissue repair, through fibroblast modulation and extracellular matrix remodeling. The data presented here indicate the lipocalin-derived peptide is an active molecule, which can be useful to develop new the therapeutic medications for healing and regeneration.

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