Amino Acids (2015) 47:317–328
DOI 10.1007/s00726-014-1860-6

ORIGINAL ARTICLE

Wound healing activity of a collagen-derived cryptic peptide

Pradipta Banerjee · Lonchin Suguna · C. Shanthi

Received: 25 August 2013 / Accepted: 21 October 2014 / Published online: 11 November 2014

Abstract Wound healing involves a well-controlled series of interactions among cells and several mediators leading to the restoration of damaged tissue. Degradation of the extracellular matrix (ECM) protein collagen during remodelling of wound tissue leads to the release of bioactive peptides that can possibly influence the healing process. The RGD-containing, antioxidative collagen peptide E1 isolated in an earlier work was screened in this study for its ability to influence multiple steps of the wound healing process. E1 was assayed for and found to be chemotactic. Excision and incision wounds were created on separate groups of rats and E1 was administered topically. The wound tissues were isolated on the 4th and 8th days post-wound and subjected to biochemical and biophysical analysis. A significant decrease in lipid peroxides in the treatment group confirmed the in vivo antioxidant capacity of E1. The treatment group also displayed significant increase in total protein, collagen and amino sugar synthesis indicating faster ECM formation. The significantly increased rate of wound contraction and reepithelialisation along with higher tensile strength of the wound tissue corroborated the results of biochemical analysis. The results confirm the significant role played by collagen peptides in accelerating the healing process and justify their possible use as a pharmaceutical agent.

Keywords Dermal wound healing · Bioactive peptide · Antioxidant · Collagen · Chemotactic

P. Banerjee · C. Shanthi (✉)
School of Bio Science and Technology, VIT University,
Vellore 632014, Tamil Nadu, India
e-mail: cshanthi@vit.ac.in

L. Suguna
Department of Biochemistry, Central Leather Research Institute,
Chennai 600020, Tamil Nadu, India

Introduction

A wound is defined as an injury due to an external violence, usually resulting in rupture of tissue, integument or mucous membrane. The complete wound healing process is a complex and dynamic series of events restoring cellular structures that begins at the moment of injury and can continue for months to years. It involves four overlapping steps termed as hemostasis, inflammation, proliferation and remodelling, each with carefully regulated multiple substeps that exhibit various interdependent relations.

The first phase, hemostasis, begins as an immediate response to an injury. In this phase, the aggregation of platelets leads to the formation of a blood clot, temporarily sealing the wound (Mutsaers et al. 1997; Sherratt and Dallon 2002). During the second phase (the inflammation phase), factors released by the platelets recruit neutrophils and macrophages to the wound site. Along with resident mast cells, these newly arrived cells initiate destruction of invading microorganisms and clear the cellular debris in the wound bed (Traci 2008). In the third stage (the proliferative stage), hormones released by leukocytes induce endothelial cell and fibroblast migration towards the centre of the wound leading to formation of new blood vessels and granulation tissue (Mutsaers et al. 1997). Fibroblasts further stimulate migration and proliferation of keratinocytes at the wound edge, resulting in complete coverage of the wound by a neoepidermis. In the remodelling phase, newly deposited collagen molecules are cross linked and oriented, leading to increase in the tensile strength of the tissue (Schäfer and Werner 2008; Ramos et al. 2011).

The rate of wound healing is dependent on effective synchronization of the phases (Schultz and Wysocki 2009). A chemical or biological agent with the ability to influence multiple phases of the healing process could provide the

required synchronization and result in lower healing time periods. Earlier studies report that collagen, a major protein component of the extra cellular matrix (ECM), harbours several bioactive segments that when proteolytically excised or 'exposed' through conformational modification, display novel physiological activities (Banerjee et al. 2012). Collagen type I has recently been found to be a host for such concealed peptides that exhibit diverse biological activities including angiotensin 1-converting enzyme (ACE)-inhibition, anti-angiogenesis, tumour growth inhibition, chemotaxis and oxidative stress relief.

In our previous work (Banerjee et al. 2012), we have identified a cryptic, RGD-containing bioactive peptide E1 from bovine Achilles tendon collagen exhibiting strong in vitro antioxidant activity. The peptide has been derived from the position 1,066–1,101 of collagen α-1(I) chain [Uniprot entry: P02453 (CO1A1_BOVIN) (http://www.uniprot.org/uniprot/P02453)] and it displays a molecular mass of 3.2 kDa and the sequence:

GETGPAGPAGPIGPVGARGPAGPQGPRGDKGETGEQ

The peptide is prevalent among collagen types I and II of most mammals including *Homo sapiens*, *Rattus norvegicus*, *Canis lupus familiaris*, *Mus musculus*, *Xenopus laevis*, etc., with the 12-residue C-terminal sub-sequence GPQGPRGDKGET common for all. In most of the species, the peptide is positioned in the vicinity of the C-terminal telopeptide of collagen type I, specifically near the region constituting residue numbers 1,060–1,100.

In recent years, a number of bioactive therapeutic peptides with significant in vitro activity have been identified from a wide variety of proteins, including collagen. However, a peptide with pronounced in vitro bioactivity may be unable to display similar activity when applied in vivo. Possible reasons for loss in activity include rapid digestion by proteases, modification by the target cells or conformational instability of the peptide in physiological environment. A bioactive peptide may be considered useful or truly bioactive when it produces at least similar, if not enhanced results when tested in an in vivo model. This study was, therefore, undertaken to evaluate the activity of the possibly multifunctional cryptic peptide E1, isolated from bovine tendon collagen, as an in vivo wound healing agent.

Materials and methods

Animals

Male albino Wistar strain rats in the weight range of 500–600 g and 10 months of age were used for this study. The animals were maintained in clean, sterile, polyvinyl cages and fed with commercial rat food from M/s Hindustan Lever Ltd, India [mixed with wheat flour in the ratio 1:1 (w/w)]. Food and water were provided ad libitum to the animals. All processes were carried out according to the stipulations of the Institutional Animal Care and Use Committee (IACUC).

Chemicals

Superdex 30 prep grade, pepsin, L-hydroxyproline, glucuronic acid, HEPES buffer {2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid} and chloramine-T were obtained from Sigma, St. Louis, USA. *p*-Dimethyl aminobenzaldehyde and Folin's phenol reagent were obtained from SD Fine Chemicals Limited, Mumbai, India. Plates and reagents for chemotactic assay were procured from Cell Biolab, San Diego, USA. Methyl cellosolve was obtained from E. Merck, Darmstadt, Germany. All other reagents were of analytical grade.

Isolation of E1

The peptide E1 was isolated from collagen type I as described earlier (Banerjee et al. 2012). Briefly, purified bovine tendon collagen was fragmented by microbial enzyme treatment and the hydrolysate subjected to ion-exchange chromatography followed by gel permeation chromatography. The peptide E1 was further purified by running in a 1.4 × 18 cm superdex P30 column and desalted in a 1.4 × 10 cm ephadex G10 column. The purified and desalted peptide was lyophilized with a Micro Modulyo-230 freeze-drier (Thermo Scientific, USA) and stored at −70 °C in a sealed vial until use.

Chemotactic assay of E1

The chemotactic properties of E1 were measured by a trans-well cell migration assay (Augustin 2004). Blood was collected from a healthy donor and the leukocytes isolated by the use of Hi-Sep LSM (from HiMedia, India). For the first assay, concentration of the chemotactic agent was varied while keeping the cell number constant. Different concentrations of E1, ranging from 1 to 1000 nM was dissolved in 150 µl Hank's balanced salt solution (HBSS) along with 150 mM HEPES buffer and added to the feeder tray wells. 1×10^4 cells suspended in 100 µl of HBSS were added to the membrane chamber and the cover replaced. The plates were incubated for 3 h in a 5 % CO_2 chamber at 37 °C. The number of cells migrated was measured by dislodging cells from the bottom of the permeable support and the feeder chambers followed by MTT {(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay in a new 96-well plate. Absorbance was measured at 550 nm with reference at 630 nm in a Bio-Rad microplate reader.

The chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (FMLP) and bovine serum albumin (BSA) were used as the positive and negative control, respectively. For the second assay, 100 nM of E1 and trypsin degraded E1 (1:50 enzyme: substrate ratio) were used as chemotactic agents and the cell count in the top chamber was varied from 0.1 to 50 $\times$ 10^4 per well. The amino acid sequence of E1 was uploaded to the virtual peptide cutter (http://www.web.expasy.org/peptide_cutter/) at http://www.expasy.org/ to check for trypsin cleavage sites.

A checkerboard assay was conducted with differing amounts of peptide E1 loaded in the upper and lower chambers along with a fixed number of cells (1×10^4) added to the top chamber to confirm the chemotactic nature of the peptide.

Wound creation and sample application

A 2 cm^2 full-thickness open excision wound was made in the back of 36 rats following procedures mentioned in Panchatcharam et al. (2006). The animals were divided into control and treatment groups. The peptide was dissolved in physiological saline to a final concentration of 60 µM and 200 µl of this was applied topically to the test rats, once daily for a period of 12 days. 200 µl of physiological saline was applied to the control rats for a similar duration of time. Six rats were killed at different time intervals. The wound tissues were removed on the 4th and 8th days post-wound infliction and used for biochemical analyses.

For incisional wound creation, 12 rats were divided into two groups of six; they were anaesthetized and two paravertebral long incisions of 5 cm were made through the skin and cutaneous muscles at a distance of 1.5 cm from the vertebra. After wiping the wound dry, intermittent sutures were applied via a surgical nylon thread and a curved needle (no. 11), 1 cm apart. For the test rats, 60 µM of the peptide solution was added to the wound on a daily basis whereas the control group received same volume of saline.

Biochemical estimations

Protein from wet granulation tissues was extracted with 5 % trichloroacetic acid as per Porat et al. (1980) and was estimated by the method of Lowry et al. (1951). For collagen and hexosamine estimation, the tissue samples were defatted in chloroform: methanol (2:1) and dried in acetone before use.

Collagen was quantified using the method of Woessner (1961). The tissue was hydrolyzed in a sealed container for 15 h using 6 M HCl and the resultant hydrolysate was neutralized with NaOH before the assay. Next, the hydrolysate was mixed with the following: 3×10^{-3} g ml^{-1} Chloramine T, methyl cellosolve and acetate buffer (pH 6) in a 2:3:5 ratio followed by perchloric acid and 5 % *p*-dimethylaminobenzaldehyde in n-propanol. The resultant solution was incubated at 60 °C, cooled and absorbance measured at 577 nm. A calibration curve was plotted with standard hydroxyproline and the amount of collagen was calculated using the equation: % collagen = % hydroxyproline $\times$ 7.46.

Hexosamine was quantified using the method of Elson and Morgan (1933). To 1 ml of the hydrolysate, 1 ml of acetylacetone in 0.5 N Na_2CO_3 was added and the mixture heated for 20 min. After cooling at room temperature, 5 ml ethanol and 1 ml of *p*-dimethyl aminobenzaldehyde dissolved in a 1:1 volume mixture of HCl and ethanol were added and the absorbance measured at 530 nm. The hexaosamine content was determined using a calibration curve determined using standard glucosamine.

Ascorbic acid was estimated according to Omaye et al. (1979). The tissue was homogenised and ascorbic acid extracted in acetic acid. The extract was mixed with phosphotungstic acid reagent and absorbance measured at 700 nm.

Lipid peroxide levels were measured by the thiobarbituric acid method (Santos et al. 1980). To 0.1 ml of the acid-hydrolyzed and water-reconstituted tissue, 0.9 ml of 10 % tricloroacetic acid and 2 ml of 0.67 % thiobarbituric acid reagent were added and the resultant solution was boiled in a water bath for 20 min. After cooling at room temperature, the samples were diluted with distilled water, vortexed and centrifuged to obtain a clear solution. The pink colour developed was read at 532 nm.

Biophysical parameters

The period of epithelialization was taken as the number of days for shedding of eschar without any raw wound left behind. The rate of wound contraction was determined by planimetric method. The surface of the wound was traced on a transparent graph paper and the area was measured manually using planimeter. The reduction in the wound size was calculated using the following formula:

$$\%- \text{ Wound contraction}-= -[(\text{difference in the area of the wound in } cm^2 \text{ between the initial and on a particular post-operative day}) \times 100]/\text{area of the wound in } cm^2 \text{ immediately after the wound excision.}$$

The tensile strength of the 12th day wound tissue was measured by the method of Vogel (1970). Skin pelts were excised from the rats and the thickness was calculated with callipers. The breaking strength was determined by measuring force–elongation curves using an Instron instrument and tensile strength was calculated from the following equation:

Tensile strength = breaking strength/cross sectional area.

The shrinkage temperature of the 12th day wound tissue was measured by the method of Borasky and Nutting (1949). A small piece of the granulation tissue was moistened with a drop of water and fixed onto a heating stage fitted with an optical microscope. The tissue was heated at a constant rate with the help of a tungsten lamp and the sample was continuously observed. The temperature at which the tissue started to shrink was noted as the shrinkage temperature.

Statistical analysis

Values given for the chemotactic assay and the biochemical and biophysical parameters are reported as mean ± standard deviation. The data were analysed for statistical significance using ANOVA for larger groups and student's *t* test for comparing two groups. *p* values less than 0.05 at a confidence level of 95 % were considered significant.

Results

Chemotactic activity of E1

As depicted in Fig. 1a, the chemotactic activity displayed by E1 was dependent on peptide concentration. The decrease in activity at higher concentrations was possibly due to the recoiling nature of collagen peptides and/or the desensitization of chemotactic receptors on the leukocytes. Maximum activity displayed by E1, although lower than FMLP ($p < 0.01$), was at 100 nM with 30 % migrated cells. Interestingly, the activity increased 1.8 times with similar amounts of the trypsin-degraded peptide. The activity was also found to be dependent on the number of cells seeded in the upper chamber, as displayed in Fig. 1b. For lower cell counts, 42–45 % cells were found to migrate but the % of migratory cells decreased somewhat with increasing cell concentrations. Fragmented E1 allowed a significantly increasing number of cells ($p < 0.05$) to migrate in comparison to the intact peptide. The activity of E1 was considerably less when compared to FMLP, probably due to the larger size of the former. Smaller peptides act as potent chemotactic agents because they are able to interact with the chemotactic receptor of the concerned cell in a far more effective manner (Laskin et al. 1986). The fact that degraded E1 supports more than twice the chemotactic activity adds further evidence to the claim. Checkerboard analysis results displayed in Table 1 exhibited a similar migratory cell count for equal concentrations of E1 in the top and bottom chambers.

The virtual peptide cutter at ExPASy identified three cleavage sites for trypsin, as depicted in Fig. 2. Complete cleavage with trypsin results in four peptides, two of which display an arginine residue at the C-terminal end. The largest fragment (1.5 kDa) obtained after trypsin treatment displays a relative % abundance of 33 % proline and 22 % glycine, four GP sub-sequences and a C-terminal arginine. The decreased size, increased amount of GP and the terminal arginine residue possibly enhanced the chemotactic properties of degraded E1.

Biochemical parameters

Table 2 depicts the collagen, total protein, hexosamine, ascorbate and linoleic acid levels on the 4th and 8th day. The data indicate a consistent difference between the test and control groups in most of the cases. Lipid peroxide (LPO) levels on the 4th and 8th day were found to be significantly lower ($p < 0.01$) for treatment group when compared to the control group. Ascorbate amounts on the 8th day were found to increase threefold for the control and 2.7-fold for E1-treated groups from their respective values on the 4th day. This difference was significant ($p < 0.05$) for the 4th day but the 8th day ascorbate levels were almost

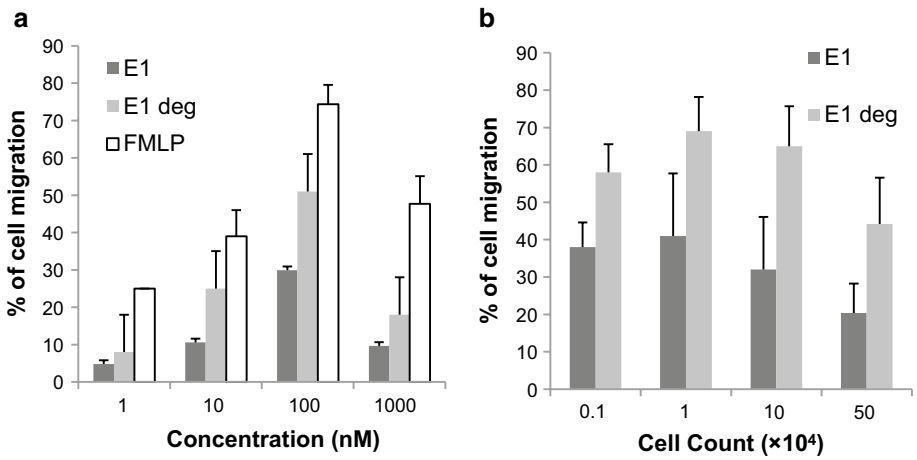

Fig. 1 Chemotactic assay with E1. **a** Results of cell migration assay with different concentrations of E1, protease degraded-E1 and FMLP. **b** Results of cell migration assays conducted with 100 nM E1 and degraded E1 with different cell counts in the top chamber. Cell count was varied from 0.1 to 50 × 10^4 cells per well

Table 1 Results of checkerboard assay conducted with varying concentrations of E1

% of cell migration				
Concentration of E1in bottom chamber (nM)	Concentration of E1 in top chamber (nM)			
	1	1	10	100
0	0	0.78 ± 0.11	0.6 ± 0.14	0.42 ± 0.14
1	3.9 ± 0.2	2.7 ± 0.86*	2.1 ± 0.78	0.71 ± 0.11
10	10.2 ± 1.1	8.8 ± 1.01	3.0 ± 1.34*	1.27 ± 0.13
100	32.8 ± 4.7	28.2. ± 2.8	1.5 ± 0.42	2.9 ± 0.68*

The asterisk mark (*) indicates the values significantly ($p > 0.05$, at 95 % confidence level) equal to each other

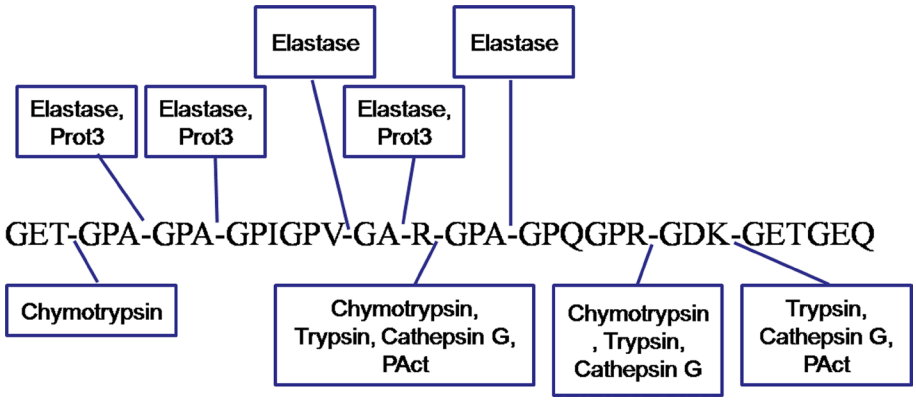

Fig. 2 Probable cleavage sites for the enzymes neutrophil elastase, proteinase 3 (Prot3), chymotrypsin, trypsin, cathepsin G and plasminogen activator (PAct) in E1 as identified through peptide cutter software

Table 2 Effect of E1 on five biochemical parameters

	Day 4	Day 8
LPO (mg/100 g body weight)		
Control	2.15 ± 0.20	4.09 ± 0.16
Test	1.57 ± 0.23[c]	0.71 ± 0.14[a]
Ascorbate (mg/100 g body weight)		
Control	2.44 ± 0.1	7.72 ± 0.75
Test	3.63 ± 0.54[c]	9.09 ± 0.69[d]
Collagen (mg/100 g body weight)		
Control	1.1 ± 0.11	2.07 ± 0.44
Test	2.6 ± 0.23[b]	5.96 ± 0.54[a]
Hexosamine (mg/100 g body weight)		
Control	0.44 ± 0.09	0.6 ± 0.08
Test	1.4 ± 0.21[b]	2.62 ± 0.16[a]
Protein (mg/100 g body weight)		
Control	1.78 ± 0.1	2.32 ± 0.43
Test	5.41 ± 0.31[a]	8.5 ± 0.56[b]

Values are expressed in mean ± SD for six animals

Level of significance expressed as [a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$ and [d] $p \geq 0.05$ as compared using *t* test at 95 % confidence level

similar; in fact, the differences between the two levels were statistically insignificant ($p > 0.05$).

On the 4th and the 8th day, the amount of collagen produced in the treatment group was 2.36 and 2.85 times that of collagen present in the control group. In both cases, the values for E1 were significantly higher ($p < 0.01$) than the control values. As noted from the table, hexosamine content was moderately high on the 4th day but the difference was significant from that of the control-treated group ($p < 0.01$). On the 8th day, hexosamine levels in the treated group increased to 4.3 times that of control group ($p < 0.01$). Protein production also followed a similar pattern. On both the 4th and 8th day, protein production was more than three times that of the control group ($p < 0.01$) suggesting an active remodelling process.

Biophysical parameters

The % wound contraction had markedly different values for the control and the test samples. As displayed in Fig. 3a, the difference was highly significant in the 4th ($p < 0.05$) and 12th ($p < 0.01$) day samples with the highest difference being on the 8th day ($p < 0.01$). Figure 3b displays the period of epithelialisation, which was found to be reduced in the test rats by a factor of 1.75 compared to that in control rats. Figure 3c displays the tensile strength of tissues from the control and treatment groups; a significant increase by a factor of 2.2 was observed for the treatment groups on the 12th day. Figure 3d presents the difference in shrinkage temperature of the collagen extracted from the control and the test tissues on the 12th day. The photographs in Fig. 4 exhibit the wound contraction for the

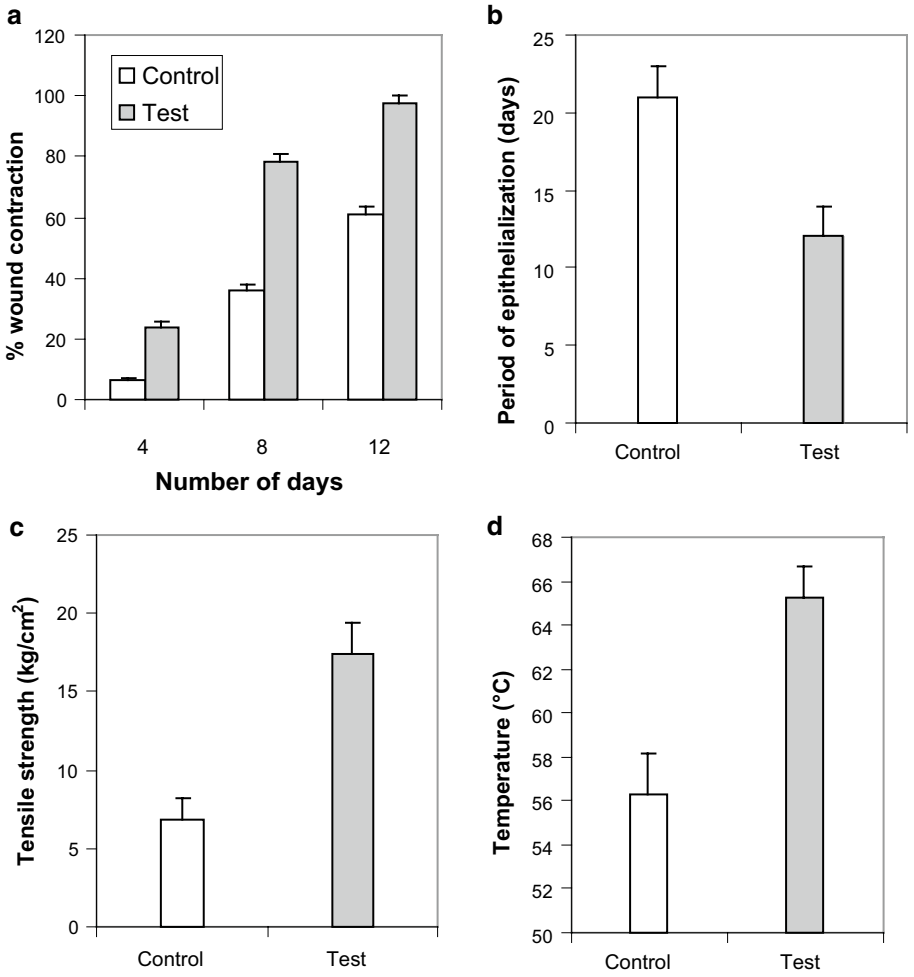

Fig. 3 Results of biophysical parameters estimation. Values are expressed in mean ± SD for six animals. **a** The % of wound contraction in control group (*white*) and treatment group (*grey*) at different days. Level of significance, as calculated by *t* test for the 4th, 8th and 12th day was $p < 0.01$ at a confidence level of 95 %. **b** Period of epithelialisation in control and treatment groups; $p < 0.01$ at a confidence level of 95 %. **c** Tensile strength of 12th day wound tissue for control and treatment groups; $p < 0.01$ at a confidence level of 95 %. **d** Shrinkage temperature of 12th day wound tissue for control and treatment groups; $p < 0.01$ at a confidence level of 95 %

0th, 4th, 8th and 12th days. The treatment group exhibited faster wound closure throughout the duration of the experiment and >90 % tissue restoration was noted on the 12th day, in comparison to 55 % restoration observed in control group.

Discussion

Activity of E1

Externally administered bioactive agents have been reported to influence one or more steps of wound healing (Balekar et al. 2012). For centuries, plant products including flavonoids and terpene-like compounds with a wide array of bioactivities including antimicrobial, haemostatic and reactive oxidative species (ROS) stress relief, have been successfully used in improving the healing process (Okoli et al. 2007). In recent years, bioactive peptides from animal proteins have also been recognized to play a key role in wound healing; a well-studied example being the human tripeptide GHK, which displays the ability to accelerate tissue remodelling (Pickart 2008). Based on the sequence of E1 and its previously reported in vitro antioxidant activity (Banerjee et al. 2012) along with its cell adhesive/migratory capabilities (Banerjee et al. 2013), the test peptide was expected to act in a multi-functional manner, ultimately influencing more than one phase in the wound healing activity cascade.

Effect on leukocyte recruitment

Fragments generated from proteolysis of collagen are known to act as chemotactic agents, resulting in recruitment of neutrophils and macrophages to the wound site. The peptides are recognized by the cell surface chemotactic receptor, followed by initiation of a series of signals that result in the cell being attracted to the wound site. Recognition of the peptide with the corresponding site in the receptor is dependent on several critical factors including peptide size, sequence and structure. Earlier studies have reported that the presence of specific amino acids, glycine, proline, arginine and phenyl alanine in peptides promote faster recognition by chemotactic receptors (Pfister et al. 1995; Afonso et al. 2013). E1 has a comparatively higher relative

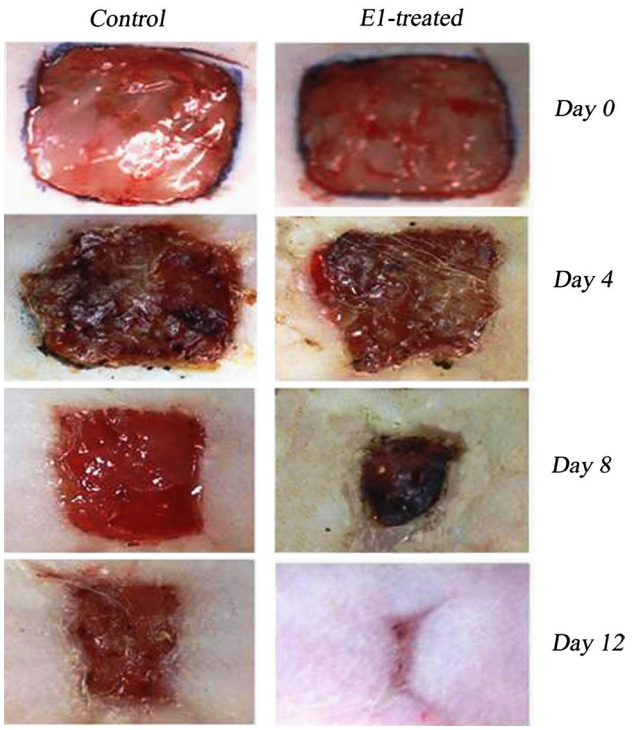

Fig. 4 Photographical representation of wound closure in control and test rats for 12 days

% occurrence of glycine (33 %) and proline (19 %) along with the presence of one arginine residue. Occurrence of an imino group imparts rigidity to the peptide and reduces torsional movements and the presence of glycine allows flexibility at certain nodes. Ample presence of both residues in the same peptide would allow for torsional rigidity at certain nodes while maintaining flexibility at other nodes, possibly leading to better interaction with chemotactic receptors. A further important criterion for a peptide to be chemotactic necessitates the presence of specific residues as the N- or C-terminal. For example, the presence of arginine as the N-terminal residue in bradykinin contributes heavily to its chemotactic properties (Kohidai et al. 2002). RGD peptides have previously been known to act as chemotactic agents (Senior et al. 1992) and the results of this study further confirm such reports.

The proteases that play a major role during the initial phases of in vivo wound healing include several matrix metalloproteases (MMP) and serine proteases such as neutrophil elastase, plasmin, proteinase 3 along with cathepsin G (Moali and Hulmes 2009). As seen in Fig. 2, the peptide E1 harboured cleavage sites for neutrophil serine proteases and the resultant fragmentation in vivo would result in several smaller fragments in the molecular weight range of 0.3–1.2 kDa. Hence, the overall in vivo chemotactic activity of E1 would depend on specific fragments obtained by proteolysis and the net chemotactic effect exhibited by the fragments.

The checkerboard analysis helps to differentiate between chemotactic and chemokinetic agents. A true chemotactic agent induces quicker cell migration upon exposure to a gradient. As seen in Table 1, equal concentrations of peptide E1 in the upper and lower compartment do not lead to a stronger migration when compared to control, based on which E1 was deduced to be a true chemotactic agent.

To summarize, E1 displayed characteristics of a moderately capable chemotactic agent leading to recruitment of a higher number of leukocytes to the affected area and providing rapid healing.

Lowering of oxidative stress

The significant lowering of ROS substantiates the effectiveness of E1 at reducing ROS-generated oxidative stress. ROS, produced by the neutrophils in the second stage, acts as a layer of defence against invading pathogens and as mediators of intracellular signalling (Wlaschek and Scharffetter-Kochanek 2005; Sen and Roy 2008) but can also lead to significant tissue damage ultimately slowing down wound healing (Steiling et al. 1999). The wound site has two distinct sources of ROS: from the respiratory burst by phagocytic cells and a continuous production of low amounts by enzymes of the NADPH oxidase family present in fibroblasts, keratinocytes and endothelial cells. While the former is involved in removing invading pathogens, the latter drives a wide array of biological processes, including gene expression and angiogenesis in the granulation phase (Roy et al. 2006). In vivo ROS scavenging is achieved by a group of native antioxidants that includes redox enzymes, organic molecules along with compounds derived from food; vitamin E and C, caroteinoids and phenolic compounds (Sen and Roy 2008; Bedard and Krause 2007). External antioxidative compounds like plant polyphenols added to the wound site have been reported to reduce ROS load and lead to faster healing (Balekar et al. 2012). In comparison to such plant-derived compounds, peptides offer unique advantages including low toxicity, higher specificity and bioavailability.

The presence of certain residues like tyrosine, phenyl alanine, tryptophan, histidine, methionine and cysteine can confer antioxidative activity to a bioactive peptide (Sarmadi and Ismail 2010). The activity arises due to the reactive nature of these amino acids; some are aromatic and others harbour lone pair of electrons which can be donated to a reactive ROS. E1, on the other hand, primarily contains a majority of uncharged residues such as glycine, proline and alanine along with few charged amino acids. Recent data support the theory that presence of sequences like GP and QG along with charged amino acids in a peptide confer it with ROS quenching properties, although the mechanism differs from that of the ‘classical’ antioxidative

residues (Sheih et al. 2009). E1 has six GP sequences, one QG sequence along with charged amino acids arginine, glutamine, glutamate, lysine and aspartate, which could actively participate in charge/electron transfer reactions and consequently, result in prominent antioxidant properties (Byun et al. 2009; Alemán et al. 2011).

The free forms of the amino acids, glutamine and arginine, have been known to exhibit antioxidative properties. Even the presence of lysine in an antioxidative peptide is important as it renders a 'sacrificial' attribute to it. Leukocyte-derived oxidants, HOCl and HOBr, although primarily involved in pathogen deactivation, cause collateral damage to proteins by reaction with lysine and tyrosine forming halolysines and halotyrosines. The reaction proceeds via halogenation of lysine followed by transfer of the halogen group to tyrosine leading to further formation of free radical products. In the sole presence of lysine, the oxidants form a major product, lysyl nitrile, which on account of its higher stability inhibits further formation of ROS species. Presence of lysine (and absence of tyrosine) in a bioactive antioxidant peptide could similarly lead to inhibition of further propagation of oxidant species via formation of stable lysyl nitrile (Sivey et al. 2013). Ultimately, along with the sequence and arrangement of residues, the conformation of the peptide may also play a major role in effective display of antioxidative properties.

The amount of lipid peroxidation is known to be a good indicator of the severity of the wound. The level of LPOs was found to be drastically reduced in the E1 treated rats in the 8th day tissues when compared to the controls. This is in accordance with our earlier work reporting the peptide E1 to be an efficient in vitro lipid peroxidation inhibitor (Banerjee et al. 2012). The data presented here also support another previous study which states that externally administered LPO inhibitory agents result in faster healing of wounds (Altavilla et al. 2001). Inhibition of LPO requires the peptide to be lipid-accessible. E1 displays an ample amount of hydrophobic residues with relative % occurrence of 5.6 % threonine, 11.1 % alanine along with 2.8 % each of highly hydrophobic residues, isoleucine and valine. Presence of hydrophobic residues increases the lipid-solubility of E1, ensuring a higher accessibility to the LPO radicals.

Vitamin C is a cofactor for the enzymes prolyl and lysyl hydroxylases, two key enzymes important for collagen synthesis. It is also a powerful natural antioxidant on its own used in quenching free radicals, chelating Fe(II) and often helping in the regeneration of other natural antioxidants, including vitamin E (Guo and DiPietro 2010). Shah et al. (1971) have reported an increase in ascorbate almost to fourfold during the differentiation phase of wound healing. The increase in ascorbate levels on the 4th day was more for E1-treated group but by the 8th day, both groups exhibited almost similar levels. Ascorbate also plays a role in collagen synthesis apart from being a general antioxidant. E1 being a moderately powerful antioxidant shared some of the ROS load along with native ROS scavengers in the treated group. The control group, due to lack of an externally added antioxidative agent, would be dependent solely on naturally available antioxidative molecules including ascorbate. This would ultimately lead to an increase in the amount of ascorbate accumulated, as was found in the study.

Effect on fibroblast recruitment and wound contraction

Integrins are a large family of cell surface glycoproteins that mediate cell–matrix adhesion. Their tissue distribution is huge and almost all the cells involved in wound healing, including neutrophils, keratinocytes, endothelial cells and fibroblasts, display one or the other sub-type of integrin (Jokinen et al. 2004). The RGD sequence is considered to be a classical integrin-recognition motif used by several ECM components, fibronectin in particular, for cell recruitment (Ruoslahti 1996). A large fraction of the total number of cell-recruitment motifs in native collagen remains inaccessible to the receptors (Ruoslahti 1996). However, in degraded collagen, the motifs are 'exposed' or it undergoes a conformational change, thereby allowing it to interact with integrin receptors and initiate cell adhesion (Eliceiri and Cheresh 1999).

Peptides from fibronectin and collagen are known to effect migration of various cells. For example, vascular smooth cells stimulated with platelet derived growth factor migrate relatively faster on collagen type I fragments than on intact collagen. This is due to a switch in integrin expression from collagen-binding integrins to αvβ3-integrins upon exposure to the peptides. In a similar fashion, MMP cleavage of collagen type IV exposes a fragment that results in decreased binding of endothelial cells to collagen receptor α1β1 and increased binding to αvβ3 (Stringa et al. 2000; Xu et al. 2001). The RGD motif in E1 is a αvβ3-recognition motif (D'Souza et al. 1991) with high cell adhesion properties (Banerjee et al. 2013) and may be crucial in cell recruitment to the wound site.

One of the effects of higher migratory influx of cells is to elevate the rate of wound contraction, as noticeable in the treatment group in this study. Within the wound bed, fibroblasts produce collagen as well as two other important components of the ECM, glycosaminoglycans (GAG) and proteoglycans (PG). Hexosamine is an irreplaceable component in building GAGs and PGs and its quantification helps in determining the amount of ECM being produced during the proliferative and the remodelling stages. GAGs form a hydrated gel-like ground substance in which the collagen molecules are embedded. They are charged and can possibly influence the deposition and orientation of newly

synthesized ECM components through ionic interactions (Bastiaansen-Jenniskens et al. 2009). Increased GAG and ECM protein synthesis is indicative of increased fibroblast recruitment, signifying an active remodelling phase (Costarelli and Emery 2009). The elevated total protein levels in the treatment group also confirmed the huge ECM turnover.

During the proliferative phase, there is an abundance of fibroblasts and endothelial cells in the reparative dermis. Capillary growth, formation of granular tissue and synthesis of collagen are the primary activities at this stage (Guo and DiPietro 2010). Collagen mass in normal tissues is dependent on the balance between rates of synthesis and degradation. A rapid increase of collagen production in the treatment group is indicative of higher rate of granulation tissue formation, resulting in faster re-epithelialisation. The physical parameters, shrinkage temperature and tensile strength of the wound tissue are dependent on the degree of collagen cross linking. A higher value for both shrinkage temperature and tensile strength in treatment group confirmed the presence of cross-linked, well-arranged collagen fibres, indicating the higher fibroblast recruitment.

Overall, the data confirm that the application of the peptide had a considerable effect on collagen production and deposition, which consequently had a major influence on the remodelling phase.

Tracking and documenting the changes in the wound area can act as an accurate marker of the rate of healing and wound contraction. Cutaneous wound healing primarily occurs through two independent processes, contraction and epithelialisation. Wound contraction is the centripetal displacement of the wound edges facilitating gap closure at 5–15 days after the injury. It is a hallmark of the proliferative phase and is brought about by activated fibroblasts or myofibroblasts that migrate into the damaged tissue in response to cytokines locally released from inflammatory and resident cells. In response to the mechanical challenge, activated fibroblasts, along with depositing massive amounts of ECM acquire contractile stress fibres. This leads to establishment of a large number of cell–cell and cell-ECM networks, which is sufficient for generating the force necessary for wound contraction (Hinz et al. 2007). A faster wound contraction demands a larger population of activated myofibroblasts and consequently, higher amounts of ECM synthesis and deposition. The significant increase in wound contraction as evident in Fig. 4 for the treatment group, therefore, stems from the holistic influences of E1 in recruiting cells, reducing ROS damage and increasing collagen production.

Epithelialisation is accompanied by the migration of keratinocytes into the injured tissue. This step is defined as the reconstitution of an organized, stratified epithelial layer that permanently covers the wound, leading to restoration of functionality. The time period for epithelialisation for the treatment groups were found to be almost half of that required by the control groups, indicating faster healing. Upon completion of epithelialisation, the reconstructed ECM takes over the mechanical load and myofibroblasts perish by massive apoptosis, indicating the transition from granulation tissue to a scar tissue (Laplante et al. 2001).

To summarize, the peptide E1 could significantly affect the various biochemical and biophysical parameters at multiple levels of the wound healing process. The summary of the action of E1 is depicted in Fig. 5.

Bioavailability of E1

Collagen peptides have previously been shown to exhibit chemotactic activity (O'Reilly et al. 2009). Peptides PGP, POG and PO (where O stands for hydroxyproline) exhibit chemotactic effect on peripheral blood neutrophils and thus influence the second phase of wound healing (Shigemura et al. 2009). Even applying collagenases derived from *Clostridum histolyticum* has proven beneficial for wound healing because they degrade the collagen matrix giving rise to bioactive fragments that stimulate wound closure (Demidova-Rice et al. 2011). The complexes of peptide GHK with Cu^{2+} and biotin have displayed potential to improve all phases of wound healing viz., inflammation, fibroblast proliferation, connective tissue formation and remodelling of wound tissue (Arul et al. 2007; Pickart 2008). The pentapeptide KTTKS, a subfragment of type I collagen, has been found to be the minimum sequence necessary to stimulate collagen synthesis from fibroblasts (Katayama et al. 1993). Even the chemically modified fibronectin-derived peptide, Ac-PHSRN-NH_2, has been shown to stimulate reepithelialisation and wound closure in obese diabetic mice (Livant et al. 2000). Most of these wound healing peptides, including E1, displayed a higher % occurrence of hydrophilic residues when compared to collagen.

Bioactive peptides have a short window of action for displaying activity as they are prone to proteolytic fragmentation. The period of time it stays intact and active depends on its localization and conformation, especially on the protease cleavage sites it carries. The first series of proteases gaining importance in the wound bed stems from the presence of neutrophils and includes MMP-8, elastase, plasmin, proteinase 3 and cathepsin G, the latter known to degrade denatured collagen. E1 exhibits cleavage sites for all the proteases except MMP-8. However, degradation in this case possibly results in beneficial effects as it leads to elevated chemotaxis. In the second phase, MMPs 2, 3, 9, 7 and 12, ADAM-10 and 17 along with cathepsin L initiate the chemokine signalling. During re-epithelialization, proteases ADAM-9, 10, 12 and 17, MMP-2 and 9 along with plasmin and tolloids initiate synthesis of several growth

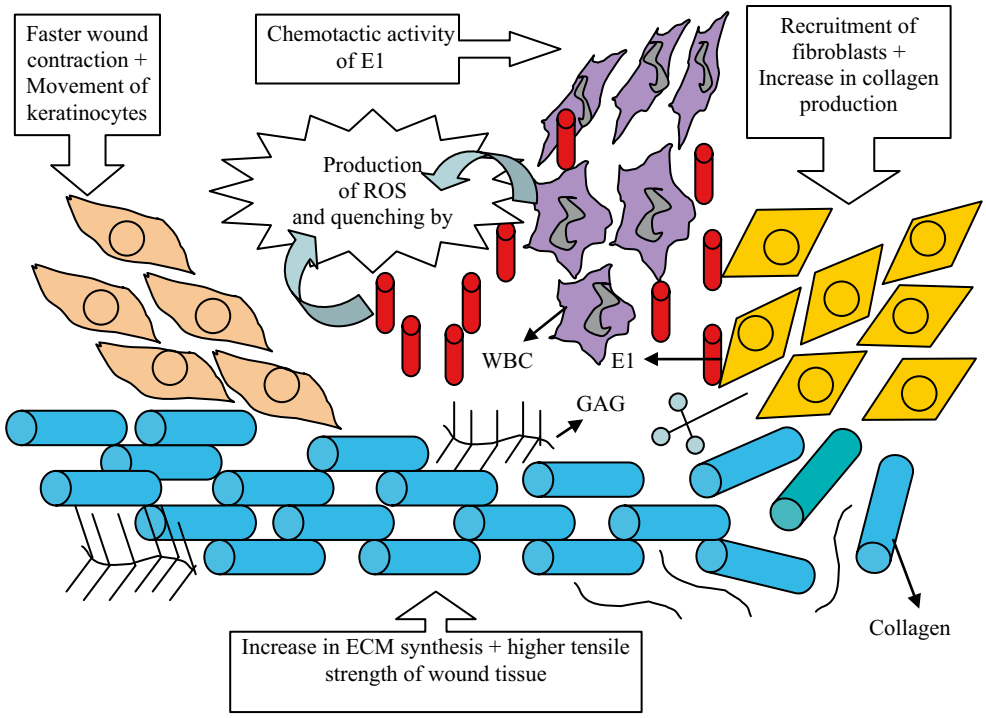

Fig. 5 Schematic representation of the action of E1 at different stages of the wound healing process. E1 initiates chemotaxis, lowers ROS stress and recruits fibroblasts leading to faster contraction and healing

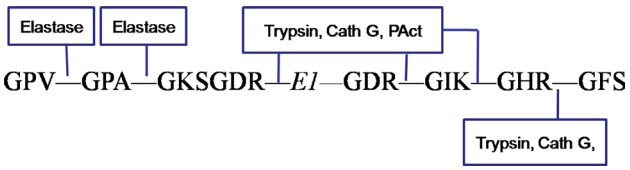

Fig. 6 Probable cleavage sites in collagen type I α chain sequences flanking the E1 peptide. The notations Cath G and PAct represent cathepsin G and plasminogen activator, respectively

factors. The protease groups, tolloids and ADAMTS, prevalent in the remodelling phase are linked intimately with ECM deposition and assembly (Moali and Hulmes 2009). Although E1 does not carry explicit cleavage sites for tolloids, ADAMs and ADAMTS, peptides can be cleaved by proteases at sub-optimal sites if it is flexible enough to fit into the active site (Clendeninn and Krzysztof 2001). On the other hand, presence of structural constraints like 33 % proline in the peptide may render it inaccessible to the protease active sites.

The pattern of cleavage sites flanking E1 in the collagen α1 chain as depicted in Fig. 6 reveals recognition sites for trypsin, cathepsin G and plasmin on both sides of E1 along with elastase cleavage site in the N-terminal flanking region. Presence of the cleavage sites makes E1 amenable to be excised in vivo during tissue remodelling. Overall, linking known enzyme-substrate reactions with observations at the cellular level and extrapolation of in vitro events to in vivo ones is difficult due to the sheer number and complexity of agents involved. However, based on the results, it would be safe to assume that a bioactive fragment such as E1 may just be one among the numerous cryptic peptides released during ECM degradation leading to modulation of multiple physiological processes.

Conclusions

The time it takes for a wound to heal depends on the particular nature of the wound, the causative agent and inherent interplay of complex factors at the wound site. An extensive range of wound dressing materials are now at the disposal of wound care practitioners. A desired wound dressing should be (1) biocompatible, (2) able to maintain a moist environment, (3) physically protect the wound against external agents and (4) most importantly be able to hasten one or more phases of the wound healing (Jones et al. 2006; Schneider et al. 2009). Collagen-based dressing materials like hydrocolloids and hydrogels fulfil most of the above listed necessities and have been proven to be beneficial (Pielesz and Paluch 2012). Collagen and its constituent bioactive cryptic peptides like E1 discussed in this study are biocompatible, non immunogenic and are less susceptible to be degraded by serine proteases by virtue of

the excess proline residues (Banerjee and Shanthi 2012). Topical applications of such bioactive molecules as a dressing applied directly on the wound could be an easy and effective way to sustain a 'local' effect and do away with the need to apply on a daily basis.

Acknowledgments This study was supported by research grant from DST, Govt. of India (SERC SL NO 1328). The authors are thankful to VIT University, Vellore, and CLRI, Chennai, where work was carried out. The authors express their gratitude to Mr. Sandeep Bhowmik, for his help in conducting some of the assays.

Conflict of interest The authors declare that they have no conflict of interest.

References

Afonso PV, McCann CP, Kapnick SM, Parent CA (2013) Discoidin domain receptor 2 regulates neutrophil chemotaxis in 3D collagen matrices. Blood 121:1644–1650

Alemán A, Giménez B, Pérez-Santin E, Gómez-Guillén MC, Montero P (2011) Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. Food Chem 125:334–341

Altavilla D, Saitta A, Cucinotta D, Galeano M, Deodato B, Colonna M et al (2001) Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis in the genetically diabetic mouse. Diabetes 50:667–674

Arul V, Kartha R, Jayakumar R (2007) A therapeutic approach for diabetic wound healing using biotinylated GHK incorporated collagen matrices. Life Sci 80:275–284

Augustin HG (ed) (2004) Methods in endothelial cell biology, 1st edn. Springer Lab Manual. Springer, Heidelberg

Balekar N, Nakpheng T, Katkam NG, Srichana T (2012) Wound healing activity of ent-kaura-9(11), 16-dien-19-oic acid isolated from *Wedelia trilobata* (L.) leaves. Phytomedicine 19:1178–1184

Banerjee P, Shanthi C (2012) Isolation of novel bioactive regions from bovine Achilles tendon collagen having angiotensin I-converting enzyme-inhibitory properties. Process Biochem 47:2335–2346

Banerjee P, Suseela G, Shanthi C (2012) Isolation and identification of cryptic bioactive regions in bovine Achilles tendon collagen. Protein J 31:374–386

Banerjee P, Mehta A, Shanthi C (2013) Screening for novel cell adhesive regions in bovine Achilles tendon collagen peptides. Biochem Cell Biol 92:9–22

Bastiaansen-Jenniskens YM, Koevoet W, Jansen KMB, Verhaar JAN, DeGroot J, Van Osch GJVM (2009) Inhibition of glycosaminoglycan incorporation influences collagen network formation during cartilage matrix production. Biochem Biophys Res Commun 379:222–226

Bedard K, Krause K-H (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313

Borasky R, Nutting JC (1949) Microscopic methods for determining shrinkage temperature of collagen and leather. JALCA 44:830–841

Byun H-G, Lee JK, Park HG, Jeon J-K, Kim S-K (2009) Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. Process Biochem 44:842–846

Clendeninn NJ, Krzysztof A (eds) (2001) Cancer drug discovery and development: matrix metalloproteinase inhibitors in cancer therapy, 13th edn. Humana Press Inc., Totowa, pp 39–57

Costarelli V, Emery PW (2009) The effect of protein malnutrition on the capacity for protein synthesis during wound healing. J Nutr Health Aging 13:409–412

D'Souza SE, Ginsberg MH, Plow EF (1991) Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. Trends Biochem Sci 16:246–250

Demidova-Rice TN, Geevarghese A, Herman IM (2011) Bioactive peptides derived from vascular endothelial cell extracellular matrices promote microvascular morphogenesis and wound healing in vitro. Wound Repair Regen 19:59–70

Eliceiri BP, Cheresh DA (1999) The role of αv integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 103:1227–1230

Elson LA, Morgan WTJ (1933) A colorimetric method for the determination of glucosamine and chondrosamine. Biochem J 27:1824–1828

Guo S, DiPietro LA (2010) Factors affecting wound healing. J Dent Res 89:219–229

Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat M-L, Gabbiani G (2007) The myofibroblast. Am J Pathol 170:1807–1816

Jokinen J, Dadu E, Nykvist P, Käpylä J, White DJ, Ivaska J et al (2004) Integrin-mediated cell adhesion to type I collagen fibrils. J Biol Chem 279:31956–31963

Jones V, Grey JE, Harding KG (2006) Wound dressings. BMJ 332:777–780

Katayama K, Armendariz-Borunda J, Raghow R, Kang AH, Seyer JM (1993) A pentapeptide from type I procollagen promotes extracellular matrix production. J Biol Chem 268:9941–9944

Kohidai L, Kovács K, Csaba G (2002) Direct chemotactic effect of bradykinin and related peptides-significance of amino- and carboxyterminal character of oligopeptides in chemotaxis of tetrahymena pyriformis. Cell Biol Int 26:55–62

Laplante AF, Germain L, Auger FA, Moulin V (2001) Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions. FASEB J 15:2377–2389

Laskin DL, Kimura T, Sakakibara S, Riley DJ, Berg RA (1986) Chemotactic activity of collagen-like polypeptides for human peripheral blood neutrophils. J Leukoc Biol 39:255–266

Livant DL, Brabec RK, Kurachi K, Allen DL, Wu Y, Haaseth R et al (2000) The PHSRN sequence induces extracellular matrix invasion and accelerates wound healing in obese diabetic mice. J Clin Invest 105:1537–1545

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Moali C, Hulmes DJS (2009) Extracellular and cell surface proteases in wound healing: new players are still emerging. Eur J Dermatol 19:552–564

Mutsaers SE, Bishop JE, McGrouther G, Laurent GJ (1997) Mechanisms of tissue repair: from wound healing to fibrosis. Int J Biochem Cell B 29:5–17

O'Reilly PJ, Hardison MT, Jackson PL, Xu X, Snelgrove RJ, Gaggar A et al (2009) Neutrophils contain prolyl endopeptidase and generate the chemotactic peptide, PGP, from collagen. J Neuroimmunol 217:51–54

Okoli CO, Akah PA, Okoli AS (2007) Potentials of leaves of *Aspilia africana* (Compositae) in wound care: an experimental evaluation. BMC Complement Altern Med 7:24

Omaye ST, Turnbull JD, Sauberlich HE (1979) Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Methods Enzymol 62:3–11

Panchatcharam M, Miriyala S, Gayathri VS, Suguna L (2006) Curcumin improves wound healing by modulating collagen and decreasing reactive oxygen species. Mol Cell Biochem 290:87–96

Pfister RR, Haddox JL, Sommers CI, Lam KW (1995) Identification and synthesis of chemotactic tripeptides from alkali-degraded whole cornea. A study of *N*-acetyl-proline-glycine-proline and *N*-methyl-proline-glycine-proline. Invest Ophthalmol Vis Sci 36:1306–1316

Pickart L (2008) The human tri-peptide GHK and tissue remodeling. J Biomater Sci Polym Ed 19:969–988

Pielesz A, Paluch J (2012) Therapeutically active dressings–biomaterials in a study of collagen glycation. Polim Med 42:115–120

Porat S, Rousso M, Shoshan S (1980) Improvement of gliding function of flexor tendons by topically applied enriched collagen solution. J Bone Joint Surg Br 62-B:208–213

Ramos R, Silva JP, Rodrigues AC, Costa R, Guardão L, Schmitt F et al (2011) Wound healing activity of the human antimicrobial peptide LL37. Peptides 32:1469–1476

Roy S, Khanna S, Nallu K, Hunt TK, Sen CK (2006) Dermal wound healing is subject to redox control. Mol Ther 13:211–220

Ruoslahti E (1996) RGD and other recognition sequences for integrins. Annu Rev Cell Dev Bi 12:697–715

Santos MT, Valles J, Aznar J, Vilches J (1980) Determination of plasma malondialdehyde-like material and its clinical application in stroke patients. J Clin Pathol 33:973–976

Sarmadi BH, Ismail A (2010) Antioxidative peptides from food proteins: a review. Peptides 31:1949–1956

Schäfer M, Werner S (2008) Oxidative stress in normal and impaired wound repair. Pharmacol Res 58:165–171

Schneider A, Wang XY, Kaplan DL, Garlick JA, Egles C (2009) Biofunctionalized electrospun silk mats as a topical bioactive dressing for accelerated wound healing. Acta Biomater 5:2570–2578

Schultz GS, Wysocki A (2009) Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen 17:153–162

Sen CK, Roy S (2008) Redox signals in wound healing. BBA Gen Subj 1780:1348–1361

Senior RM, Gresham HD, Griffin GL, Brown EJ, Chung AE (1992) Entactin stimulates neutrophil adhesion and chemotaxis through interactions between its Arg-Gly-Asp (RGD) domain and the leukocyte response integrin. J Clin Invest 90:2251–2257

Shah RV, Hiradhar PK, Magon DK (1971) Ascorbic acid in the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. J Embryol Exp Morphol 26:285–293

Sheih I-C, Wu T-K, Fang TJ (2009) Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. Bioresour Technol 100:3419–3425

Sherratt JA, Dallon JC (2002) Theoretical models of wound healing: past successes and future challenges. C R Biol dnlm 325:557–564

Shigemura Y, Iwai K, Morimatsu F, Iwamoto T, Mori T, Oda C et al (2009) Effect of prolyl-hydroxyproline (Pro-Hyp), a food-derived collagen peptide in human blood, on growth of fibroblasts from mouse skin. J Agric Food Chem 57:444–449

Sivey JD, Howell SC, Bean DJ, McCurry DL, Mitch WA, Wilson CJ (2013) Role of lysine during protein modification by HOCl and HOBr: halogen-transfer agent or sacrificial antioxidant? Biochemistry 52:1260–1271

Steiling H, Munz B, Werner S, Brauchle M (1999) Different types of ROS-scavenging enzymes are expressed during cutaneous wound repair. Exp Cell Res 247:484–494

Stringa E, Knäuper V, Murphy G, Gavrilovic J (2000) Collagen degradation and platelet-derived growth factor stimulate the migration of vascular smooth muscle cells. J Cell Sci 113(Pt 11):2055–2064

Traci AW (2008) Immune cells in the healing skin wound: influential players at each stage of repair. Pharmacol Res 58:112–116

Vogel HG (1970) Tensile strength of skin wounds in rats after treatment with corticosteroids. Acta Endocrinol 64:295–303

Wlaschek M, Scharffetter-Kochanek K (2005) Oxidative stress in chronic venous leg ulcers. Wound Repair Regen 13:452–461

Woessner JF Jr (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93:440–447

Xu J, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC, Yuen SM (2001) Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. J Cell Biol 154:1069–1079