Summary. A combination of vascular pathologies and other complicating factors results in chronic wounds which constitute a serious burden for both patients and national health systems, due to prolonged hospital stays, high costs, and prolonged nursing staff dedication. Here we investigate whether proadrenomedullin N-terminal 20 peptide (PAMP), a naturally occurring peptide of the skin with antimicrobial and proangiogenic properties, either alone or in combination with autologous skeletal muscle stem/progenitor cells, acts as a wound healing factor. The rabbit ear was chosen as a test system, since it offers a reliable model for normoxic and ischemic wounds. Topical treatments with PAMP, stem/progenitor cells, or a combination of both, resulted in significant improvements of healing, when compared to untreated wounds. PAMP was very effective in promoting reepithelialization and angiogenesis, whereas treatment with stem/progenitor cells alone resulted in less wound contraction. Interestingly, the combination of PAMP and stem/progenitor cells, while maintaining angiogenic potency, reverted to the contraction levels observed in the untreated controls. Under ischemic conditions, generalized necrosis of the dermis and the underlying cartilage was observed in untreated wounds. Treatments of these wounds with PAMP or stem/progenitor cells allowed a timely recovery. In conclusion, PAMP either alone or in combination with autologous stem/progenitor cells may provide a useful tool for improving wound healing both in normoxic and ischemic conditions.

Key words: PAMP, Wound healing, Rabbit ear model, Ischemic wounds, Stem/progenitor cells.

Introduction

Despite an improved standard of living in many countries, the world population is affected by a growing incidence of vascular diseases, which in large measure can be attributed to an inappropriate lifestyle, including poor eating habits and lack of exercise. Added to this, when we consider concomitant pathologies such as diabetes and/or cardiovascular or cerebrovascular disorders, along with an increasing life expectancy in developed countries, we are likely to encounter vascular diseases even more frequently in the clinic (Diehm et al., 2009; Lejay et al., 2009).

Vascular pathologies lead to cutaneous lesions in the lower limbs which are often complicated by ischemia. For instance, chronic venous insufficiency associated with skin wounds affects 2% of the population, and 5-10% of patients suffering arteriosclerosis present skin ulcerations (Gloviczki and Yao, 2001). The economic burden of these afflictions for health care systems is enormous. For instance, intensive treatment for a chronic wound patient in Austria costs between 1071 to 7844 euros per case (Habacher et al., 2007). It is also relevant that in the U.K., the time spent by nursing staff in caring for these types of skin lesions is calculated at 20-30% of their working time. Despite all these efforts, a Mayo Clinic study found that 66% of ulcers were not healed after 6 months of treatment (Takahashi et al., 2009).

Current topical treatments for skin wounds have led to inconsistent results in terms of promoting healing. These include the use of gels (Kim et al., 2008; Newman et al., 2008) or biological skin substitutes comprised of cells on a matrix to treat venous and diabetic ulcers (Moore et al., 2008; Ulicna et al., 2010; Liu et al., 2010). Cell culture techniques have been employed to create artificial skin with the purpose of treating chronic and
acute skin wounds and burn victims (Sood et al., 2009; Scuderi et al., 2009). Furthermore, several growth factors have been used to enhance the natural response of the organism towards this condition (Bao et al., 2009; Rozman and Bolta, 2007). In addition, application of stem cells of various origins to the treatment of wounds has attracted great interest (De Haro et al., 2009). These cells can be obtained from adipose tissue (Hong et al., 2010) or have a mesenchymal origin (Sorrell and Caplan, 2010).

Several processes are needed to ensure proper wound healing, including reepithelialization, connective tissue fiber regeneration, and angiogenesis (Moreira and Markovchick, 2007). These natural phenomena may be impaired by different conditions such as tissue ischemia, bacterial infections, and chronic inflammation, leading to a persistent wound (Guo and DiPietro, 2010). Therefore, natural or artificial compounds that improve the former processes and/or mitigate the latter may offer alternative therapeutic options for patients suffering from chronic wounds.

Proadrenomedullin N-terminal 20 peptide (PAMP) is an amidated, 20 amino acid long, regulatory peptide which, together with adrenomedullin (AM), is generated by posttranslational processing of the proadrenomedullin precursor. Both peptides exhibit similar physiological properties, including vasodilatation, hormone secretion modulation, and regulation of renal and cardiovascular functions (Lopez and Martinez, 2002). PAMP is also a potent proangiogenic factor (Martinez et al., 2004) and possesses antimicrobial activity against Gram-negative microorganisms (Martinez et al., 2006). Proadrenomedullin is expressed at high levels in the integument, especially by the keratinocytes and all the secretory cells of the skin glands (Martinez et al., 1997; Welsch et al. 2002), suggesting that AM and PAMP may be exerting a protective role at this location and might constitute a natural mechanism of response when skin wounding occurs. AM has been shown to improve wound healing in a rat model in both normal and impaired situations.

Fig. 1. Step-by-step process of the rabbit ear wound model employed in this study. Following anesthesia, a circular cut is made in the dorsal epidermis of the rabbit ear (A), the epidermis between the borders is removed (B), and the cells and/or peptides are mixed with blood from the wound and allowed to clot in situ. After treatment, the clot is protected with a plastic frame and the animal is equipped with a conical collar to avoid scratches (C).
(Martinez et al., 1999). Since PAMP is a more potent angiogenic factor than AM (Martinez et al., 2004) we hypothesized that it might be very efficient in improving wound healing.

In this study, we demonstrate that PAMP improves wound repair in a rabbit ear model under both normoxic and ischemic conditions. In addition, PAMP treatment was compared with autologous grafts of stem/progenitor cells obtained from biopsies of the rabbit’s dorsal skeletal muscle, and a combination of both treatments.

**Materials and methods**

**Animals**

Male, white New Zealand rabbits, weighting 3.3±0.2 Kg (n=30), were caged under standard light and temperature conditions, with free access to food and water throughout the study. All experimental procedures were approved by the local committee for animal welfare and were carried out in accordance with the European Community Council Directive (86/609/EEC).

**Harvest and culture of stem/progenitor cells**

Rabbits were anesthetized with an intramuscular mixture of ketamine chlorohydrate (70 mg/Kg), diazepam (1.5 mg/Kg), and chlorpromazine (1.5 mg/Kg). A biopsy (1 g) of the dorsal muscle was obtained through a small paravertebral incision which was later closed with surgical staples. Stem/progenitor cells were obtained from the biopsy as previously described (Bujan et al., 2005, 2006). Briefly, the specimen was cut into small pieces and placed in an incubator at 37°C in a 0.1% suspension of type I collagenase (10 ml/g tissue; Worthington, Madrid, Spain) for 60 min. After a brief centrifugation, the supernatant was discarded and the pellet was resuspended in 10 ml of 1% trypsin-EDTA (Invitrogen, Barcelona, Spain) and placed in a water bath at 37°C with constant shaking for 40 min. Trypsin activity was stopped by addition of 1 ml fetal bovine serum and the final suspension was filtered through a 100 µm pore size mesh to isolate individual cells. These cells were washed 3 times in minimal essential medium (MEM, Invitrogen) and then transferred to complete culture medium (Amniomax, Invitrogen) and incubated at 37°C with a 5% CO₂ atmosphere until they reached 40-50% confluence. Cells were lifted from the culture flask with 1% trypsin-EDTA and subcultured at a 1:3 ratio.

**Induction of ischemia in the rabbit ear**

The rabbit ear contains 3 main arteries, one running through the center and the others near the edges, which provide all the blood supply to this organ. Blocking one or more of these arteries results in different levels of ischemia in the ear parenchyma (Chien, 2007). In this study, we ligated the central artery at two points at the base of the ear using silk sutures (Dexon 4/0, Braun, Madrid, Spain) and the portion of the artery located between the sutures was excised to ensure complete blood flow interruption. The extent of the ischemic insult was assessed by measuring transcutaneous oxygen pressure in the ear before and after ligation and sectioning of the central artery using a TCM-4 (Radiometer Copenhagen, Madrid, Spain).

**Wound production and application of treatments**

Rabbits with normoxic (n=15) or with ischemic ears (n=15) were anesthetized as before and subjected to circular wounds in both ears, as described (Bujan et al., 2005, 2006). Briefly, a cylindrical cutting device was used to produce circular wounds, 2 cm in diameter, in the dorsal side of each ear. The wounds affected the

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**Fig. 2.** Quantification of the reepithelialization and contraction indexes in untreated rabbits (CTRL), animals treated with 100 µg PAMP (PAMP), with 4.0x10⁶ autologous stem/progenitor cells (SCR), or with both PAMP and stem/progenitor cells (PAMP+SCR). All treatments increase reepithelialization, whereas only treatment with SCR was able to reduce wound contraction. Each bar represents the mean and S.E.M. of 5 independent animals. *: p<0.05 compared to CTRL.
epidermis and underlying dermis but did not reach the perichondrium lining the ear cartilage. The wounds were covered with a fibrin bed obtained from the animal’s own blood in which either PAMP (100 µg/wound, Phoenix Pharmaceuticals, Karlsruhe, Germany), stem/progenitor cells (4.0x10^6 cells/wound), or both were incorporated. The ear was kept in a horizontal position until the fibrin clot was established and then covered with a polyethylene device that protects the wound from physical contact without touching its healing surface. After surgery, each animal was fitted with a plastic neck collar (Bouvet, Madrid, Spain) to prevent them from scratching the wounds. All wounds were evaluated 14 days after surgery (Fig. 1).

Rabbit ear wounds were treated with 4 different protocols: i) untreated controls (n=8 normoxic + 8 hypoxic), ii) PAMP (n=8 normoxic + 8 hypoxic), iii) rabbit stem/progenitor cells (n=7 normoxic + 7 hypoxic), or iv) a combination of both PAMP and rabbit stem/progenitor cells (n=7 normoxic + 7 hypoxic).

**Macroscopic analysis of wounds**

At the macroscopic level, the extent of wound contraction and reepithelialization was measured. Photographs of the wounds were taken just after producing the defect and at the time of sacrifice. Computerized image analysis (ImageJ for Windows XP, NIH Image) was used to establish the overall surface area of each wound, as well as the nonepithelialized area at the end of the experiment. These measurements were carried out by 2 independent researchers blinded to the treatment and are expressed as mean ± standard deviation.

**Microscopic evaluation**

At the end of the experiment, tissue specimens were fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. Tissue sections (5 µm-thick) passing through the center plane of each wound were

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Fig. 3. Histological sections of the rabbit ear at the end of the experiment, 14 days post surgery. Cross sections of the whole ear showing the wounded area in an untreated animal (A) and in a rabbit that received 100 µg PAMP in the wound (B). The regenerated epidermis is indicated by arrows. The cartilage layer is labeled (letter C). Bar: 1.0 mm.
Blood vessel staining with an anti-α-actin antibody (red) in the deep dermis of the regenerated wound in untreated animals (A), and in rabbits treated with $4.0 \times 10^6$ stem/progenitor cells (B), 100 µg PAMP (C), or both (D). Sections were counterstained with hematoxylin. Quantification of angiogenesis score (number of positively stained blood vessels per microscopic field) shows a significant increase in animals treated with PAMP or PAMP plus stem/progenitor cells (E). Each bar represents the mean and S.E.M. of 5 animals. *: p<0.05 compared to CTRL. Bar: 150 µm.
PAMP improves ischemic wound healing

Fig. 5. Myosin staining (red color in the dermis) in the regenerated wound of untreated animals (A), and in rabbits treated with $4.0 \times 10^6$ stem/progenitor cells (B), 100 µg PAMP (C), or both (D). Sections were counterstained with hematoxylin. Bar: 300 µm.

Fig. 6. Representative recordings of the partial oxygen pressure in the ear parenchyma before (A) and after (B) interrupting blood flow in the central ear artery. Oxygen levels were measured transcutaneously with a TCM-4 Radiometer.
stained with hematoxylin-eosin and Masson’s trichrome for morphological assessment. Other sections were subjected to immunohistochemical procedures with antibodies against α-actin or myosin (both from Sigma Chemical Co., St Louis, MO). The angiogenic score was calculated by counting the number of α-actin positive blood vessels in 25x microscopic fields. This was repeated in 6 animals per treatment, 5 sections per animal, and 10 fields per section, for a total of 300 fields per treatment. These measurements were carried out by 2 independent researchers blinded to the treatment and are expressed as mean ± standard deviation.

**Statistical analysis**

Areas of wound contraction and reepithelialization, as well as microscopical parameters, were compared...
among treatments by ANOVA followed by the Mann-Whitney U test. Differences with p<0.05 were considered statistically significant.

**Results**

**Effects of PAMP and stem cells in normoxic wounds**

Rabbit ear wounds were treated with 4 different protocols: i) untreated controls, ii) PAMP, iii) rabbit stem/progenitor cells, or iv) a combination of both PAMP and rabbit stem/progenitor cells. Reepithelialization analysis shows that treatments with PAMP, stem/progenitor cells, or the combination of both are all significantly superior to untreated wounds, while there are no significant differences among these 3 treatments (Fig. 2A). When the percentage of wound contraction was measured at the end of the experiment, we observed that stem/progenitor cell treatment resulted in a significantly lower rate of contraction when compared to untreated wounds, whereas wounds treated with PAMP or PAMP plus stem/progenitor cell combination had similar levels of contraction to the untreated controls (Fig. 2B).

Histological observations of the wounds confirmed a better reepithelialization for the treated wounds when compared to untreated (Fig. 3). As expected, the dermis of wounds treated with PAMP showed a marked increase in new blood vessels that was higher than the angiogenesis elicited by stem/progenitor cells which, in turn, was higher than that found in untreated controls (Fig. 4). Myosin staining was reduced in wounds that had been treated with stem/progenitor cells alone when compared to all other groups, providing a good correlate with the macroscopical observation of less contraction for this treatment (Fig. 5).

**Generation of ischemia in the ear parenchyma**

To demonstrate that blockage of the ear central
artery results in significant ischemia, the oxygen content of the ear parenchyma distal to the blockage point was measured before and after the surgical intervention. In the normal ear, oxygen tension reaches 48.37±9.63 Hg mm (Fig. 6A) whereas following interruption of the central artery the oxygen content diminishes to 23.47±5.87 Hg mm (Fig. 6B), thus confirming the suitability of this model to study ischemic wounds.

Effects of PAMP and stem/progenitor cells in ischemic wounds

Ischemic wounds were exposed to the same treatments as normoxic wounds. An outstanding difference with the normoxic model consists in the almost complete lack of healing observed in the untreated wounds. In fact, one of the untreated wounds resulted in a generalized necrosis of the underlying cartilage (Fig. 7) and clear manifestations of chronification, such as persistent leukocyte infiltration and generalized fibrosis was observed in all the untreated hypoxic wounds. Against this backdrop, treatments with PAMP, stem/progenitor cells, or both, were highly significantly better than untreated wounds when reepithelialization was studied (Fig. 8A). We should notice that untreated ischemic rabbit ears were able to cover only about 40% of the wounded area (Fig. 8A), whereas under normoxic conditions they covered more than 70% in the same time period (Fig. 2A). As with normoxic wounds, treatment with stem/progenitor cells alone offered the only scenario where contraction was lower than in the other groups (Fig. 8B). Contraction was also lower in ischemic wounds than in their normoxic counterparts (Fig. 2B). Histological analysis again showed a high level of angiogenesis in the wounds treated with PAMP (Fig. 9) and lower levels of

Fig. 10. Myosin staining (red color in the dermis) in the regenerated ischemic wounds of untreated animals (A), and in rabbits treated with 4.0x10^6 stem/progenitor cells (B), 100 µg PAMP (C), or both (D). Sections were counterstained with hematoxylin. Bar: 150 µm.
myosin staining in wounds treated with stem/progenitor cells alone (Fig. 10).

**Discussion**

In this study we have shown that treatments with PAMP, autologous skeletal muscle stem/progenitor cells, or a combination of both, greatly improve wound healing whether in a normoxic or ischemic setting.

PAMP is highly expressed by the skin of humans (Martínez et al., 1997) and other mammals (Welsch et al., 2002), where it seems to play a protective role against invading microbes while maintaining skin homeostasis. Here we show that PAMP may be playing an additional role when a wound is produced in the skin. In this scenario, PAMP would be secreted from keratinocytes and skin glands surrounding the wound and would improve reepithelialization and angiogenesis, as well as protecting the healing wound from bacterial infections. In addition, it has been shown that expression of the proadrenomedullin gene is activated under hypoxia through a HIF-1 dependent mechanism (Garayoa et al., 2000), thus we expect that AM and PAMP expression would be increased in ischemic skin and dermis, leading to improved healing. Obviously, in chronic wounds the beneficial effects of intrinsic PAMP are not enough and external addition of this factor may be needed. The fact that PAMP is a naturally occurring peptide of the skin makes it especially attractive for clinical skin applications.

Several sources of autologous stem/progenitor cells are available for their potential grafting into chronic wounds. We have chosen stem/progenitor cells derived from skeletal muscle biopsies because they seem the natural choice for producing most of the cell types required for wound healing. These cells, also known as satellite cells, are abundant in the muscles and are easily identified by their single nucleus and undifferentiated aspect (Biressi and Rando, 2010). They express a particular set of markers which include m-cadherin, CD34, and Myf-5 (Bujan et al., 2005). Since they have a mesodermic origin, satellite cells can easily differentiate into muscle cells, myofibroblasts, endothelial cells, and pericytes (Wu et al., 2010), making them an excellent choice for regenerating the damaged dermis. Nevertheless, other stem cells of the mesodermic lineage, i.e. stem cells of the adipose tissue, may provide alternative sources for autologous grafts (Hong et al., 2009).

Although treatments with both PAMP and stem/progenitor cells provided clear advantages for wound healing, some differences in their mechanism of action were observed. First, PAMP was more efficient at inducing angiogenesis in the wounded dermis, a characteristic that may be extremely relevant when dealing with ischemic wounds. On the other hand, treatment with stem/progenitor cells alone resulted in less contraction of the wound, a desirable effect when trying to avoid disfiguring scars (Yagmur et al., 2010).

Nevertheless, in chronic wounds, a stronger contraction might be a benefit, since the approximation of the lesion lips may help in speeding reepithelialization and wound resolution. Interestingly, application of PAMP on top of the stem/progenitor cells prevented this phenomenon, reverting to the contraction values observed in the untreated controls. Recently, a clear link has been established between the proadrenomedullin gene and the proliferation and differentiation of stem/progenitor cells of neural origin (Vergano-Vera et al., 2010). It is possible that PAMP, which is applied in our model at the same time as the skeletal muscle stem/progenitor cells, may have a direct effect on these cells and modify their behavior on the wound environment. Unfortunately, so far no-one has investigated the relationships between PAMP and skeletal muscle stem/progenitor cells, and until this happens we can only hypothesize on this realm.

The fact that stem/progenitor cells increase reepithelialization but not wound contraction or angiogenesis whereas PAMP elicits a response in the 3 parameters may indicate that these treatments influence different cell populations of the wound environment. While stem/progenitor cells may help exclusively in epithelial cell proliferation and migration, PAMP might have binding sites in myofibroblasts and endothelial cells as well as in epithelial skin cells.

Here we have shown that application of PAMP and/or stem/progenitor cells in suspension clearly improves wound healing, especially in ischemic situations. Recent advances in tissue engineering are providing an increasing number of options, where novel biomaterials are used to build biofunctionalized scaffolds that provide optimized conditions for the attachment of stem cells and the rational release of regulatory molecules (Zaulyanov and Kirsner, 2007; Macri and Clark, 2009). Based on the present results, our group is developing a high-tech re-absorbable wound dressing that would offer a temporal environment for the incorporation of the stem/progenitor cells while liberating the right amounts of PAMP to ensure effective wound recovery.

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**Conflict of interest.** Alfredo Martínez is inventor of patents on the proangiogenic and antimicrobial properties of PAMP (U.S. Patent No. 09/931,700; WO2004/043383 A2). The remaining authors have nothing to disclose.

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