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(54) Title: TREATMENT AND COMPOSITION FOR WOUND HEALING

(57) Abstract: A method and medicament for promoting wound healing in a subject is disclosed. The medicament comprises an effective amount of an agent comprising one or more of; (i) an activated protein C (APC), (ii) a functional fragment of an APC, (iii) an APC mimetic compound, and (iv) protein C. Delivery systems including gels, sponges, gauzes and meshes incorporating the agent for topical administration are also described.

TREATMENT AND COMPOSITION FOR WOUND HEALING

Field of the Invention:

5 The present invention relates to the field of medicine and, more particularly, to wound healing and methods and compositions for promoting wound healing. In a particular application of the present invention, activated protein C (APC) is administered to a slow healing wound by, for example, topical application.

10 **Background to the Invention:**

 Wounds are internal or external bodily injuries or lesions caused by physical means, such as mechanical, chemical, viral, bacterial, or thermal means, which disrupt the normal continuity of structures. Such bodily injuries include contusions, wounds in which the skin is unbroken, incisions, wounds in which the skin is broken by a
15 cutting instrument, lacerations, and wounds in which the skin is broken by a dull or blunt instrument. Wounds may be caused by accidents or by surgical procedures.

 The healing of wounds is a complex process involving a number of stages. These include; 1) coagulation, which begins immediately after injury; 2) inflammation, which begins a few minutes later; 3) a migratory and proliferative process (granulation
20 stage), which begins within hours to days; and 4) a remodelling process with subsequent development of full strength skin (1-3).

Coagulation and Inflammation

 Coagulation controls haemostasis and initiates healing by releasing a variety of growth factors and cytokines from degranulated platelets. During the inflammation
25 phase, platelet aggregation and clotting form a matrix which traps plasma proteins and blood cells to induce the influx of various types of cells. Neutrophils are the first cells to arrive and function to phagocytise contaminating bacteria, digest the fibrin clot and release mediators to attract macrophages and activate fibroblasts and keratinocytes (3). Macrophages digest pathogens, debride the wound and secrete
30 cytokines/growth factors (eg interleukin-1 (IL-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF- β), and basic fibroblast growth factor (bFGF)) that stimulate fibroblasts and endothelial cells. Overall, the inflammatory stage is important to guard against infection and promote the migratory and proliferative stages of wound healing.

35 **Granulation and remodelling of the extracellular matrix**

 These stages include cellular migration and proliferation. Although lymphocytes and macrophages are involved, the predominant cell types are epithelial,

fibroblast and endothelial. Within hours of an injury, an epidermal covering, comprised mainly of keratinocytes, begins to migrate and cover the epidermis, a process known as re-epithelialisation. When they completely cover the wound they differentiate and stratify to form a new epidermis with a basal lamina. Angiogenesis (ie the formation of new blood vessels) occurs during this stage and provides nutrients for the developing tissue to survive. Fibroblasts migrate into the wound site and produce collagen and proteoglycans which ultimately give the wound tensile strength. As the remodelling phase progresses, granulation tissue is replaced by a network of collagen and elastin fibers leading to the formation of scar tissue.

Failed wound healing

10 Impaired dermal wound healing and/or dermal ulcers occur in patients with peripheral arterial occlusive disease, deep vein thrombosis, diabetes, pressure sores and burns (4). Despite intense investigation, the molecular mechanisms associated with impaired wound healing are poorly understood.

Wound healing is affected by numerous factors, including local factors (eg growth factors, edema, ischemia, infection, arterial insufficiency, venous insufficiency or neuropathy), systemic factors (eg inadequate perfusion and metabolic disease) and other miscellaneous factors, such as nutritional state, exposure to radiation therapy and smoking.

Leucocytes, particularly neutrophils, and macrophages persist in the surrounding tissue and secrete a range of proteases, including matrix metalloproteinases (MMPs) and serine proteases (5). Excessive accumulation of these enzymes interferes with the matrix remodelling (6). It is thought that agents which inhibit proteases will benefit wound healing (7). Another feature of some chronic wounds is the reduction or absence of angiogenesis, which prevents nutrients from accessing the newly formed tissue (8).

Existing technologies to improve wound healing

25 Chronic wounds are initially managed by treatment comprising eschar debridement, antibiotic treatment where appropriate, and regular dressing (2). Other dressings, such as hydrogels, hydrocolloids, or alginates, may also be used. Venous ulceration is treated by compression therapy, whereas arterial or diabetic ulcers require regular changes of dressings. Pressure sores are encouraged to heal by the relief of pressure at the injury site. Some other physical devices such as laser treatment, hyperbaric oxygen and electrical stimulation for arterial ulcers, are also used to promote wound healing (2, 9, 10).

For wounds that are unresponsive to such interventions, the use of tissue-engineered skin, such as Dermagraft or Apligraf, is an option. This therapy acts to prevent bacterial infection and allows the wound the chance to heal by normal reparative processes (11, 12). The use of such skin replacements to accelerate wound healing depends on the availability of an existing vascular supply in the existing wound.

Another approach to wound healing involves the administration of growth factors/ cytokines, which have been shown to accelerate cell proliferation *in vitro* and/or to promote wound healing in some animal models. These include IL-1, platelet-derived growth factor (PDGF), EGF, VEGF, TGF- β , and bFGF (2). Procuren (Curative Technologies), an autologous platelet releasate, contains at least five growth factors, that aid in the formation of granulation tissue and re-epithelialisation. This autologous growth factor mix has achieved some success in human subjects with ulcerated limb lesions (13). However, on the whole, results from most clinical trials using growth factors/ cytokines have been disappointing. For example, EGF failed to heal venous stasis ulcers and IL-1 failed to treat pressure sores effectively (2). Similar results were reported using bFGF (14). The reason for the lack of efficacy is not certain, but may relate to the multifactorial effects, some undesirable for healing, of growth factors/ cytokines.

Thus, there is an ongoing need to identify and develop new agents for the promotion of wound healing.

Activated protein C (APC) is a serine protease having a molecular weight of about 56 kD that plays a central role in physiological anticoagulation. The inactive precursor, protein C, is a vitamin K-dependent glycoprotein synthesised by the liver and endothelium and is found in plasma. Activation of protein C occurs on the endothelial cell surface and is triggered by a complex formed between thrombin and thrombomodulin (15, 16). Another endothelial specific membrane protein, endothelial protein C receptor (EPCR), has been shown to accelerate this reaction more than 1000-fold (17). Endothelial APC functions as an anticoagulant by binding to the co-factor, protein S, on the endothelial surface, which inactivates the clotting factors Factor VIIIa and Factor Va. The importance of APC as an anticoagulant is reflected by the findings that deficiencies in this molecule result in familial disorders of thrombosis (18).

Recently, it has also been reported that APC additionally acts as an anti-inflammatory agent and directly activates the protease, gelatinase A (17, 20). Gelatinase A is secreted by many different cell types, including smooth muscle cells, fibroblasts and endothelial cells. By degrading the collagens present in the basement membrane (21) and allowing cells to invade the stroma, gelatinase A plays an important role in physiological remodelling and angiogenesis (22). Gelatinase A also plays an important role in numerous diseases, such as promoting the invasion of thymic epithelial tumors (23), promoting the destruction of the joint in arthritis by cleaving collagen from the cartilage matrix (24) and contributing to cardiac mechanical dysfunction during reperfusion after ischemia (25). In addition to its ability to degrade the matrix, gelatinase A can also target other substrates. For example, it cleaves big endothelin-1 to yield a potent vasoconstrictor, implicating gelatinase A as a regulator of vascular reactivity (26). Gelatinase A release can also mediate platelet aggregation (27).

Further, and as is demonstrated in the examples provided hereinafter, APC is also able

to promote regeneration of endothelial cells after wounding *in vitro*, stimulate re-epithelialisation, fibroblast invasion and angiogenesis in a chicken embryo and enhance wound healing in a rat wounding model. These functions when taken together with the abovementioned anticoagulating, anti-inflammatory and Gelatinase A-activating functions, strongly indicate that APC, functional fragments thereof, and the precursor of APC (ie protein C) is/are useful for the treatment of wounds and, particularly, slow-healing wounds.

Summary of the Invention:

Thus, in a first aspect, the present invention provides a method for promoting wound healing in a subject, said method comprising administering to said subject an effective amount of an agent comprising one or more of;

- (i) an APC,
 - (ii) a functional fragment of an APC,
 - (iii) an APC mimetic compound, and
 - (iv) protein C,
- optionally in admixture with a pharmaceutically-acceptable carrier.

In a second aspect, the present invention provides a medicament for promoting wound healing in a subject, said medicament comprising an amount of an agent comprising one or more of;

- (i) an APC,
 - (ii) a functional fragment of an APC,
 - (iii) an APC mimetic compound, and
 - (iv) protein C,
- in admixture with a pharmaceutically-acceptable carrier.

In a third aspect, the present invention provides a delivery system (eg a gel, sponge, gauze or mesh) incorporating an amount of an agent comprising one or more of;

- (i) an APC,
- (ii) a functional fragment of an APC,
- (iii) an APC mimetic compound, and
- (iv) protein C,

said delivery system being suitable for application to a wound and thereafter promoting wound healing.

In a fourth aspect, the present invention provides the use of an agent comprising one or more of;

- (i) an APC,
- (ii) a functional fragment of an APC,
- (iii) an APC mimetic compound, and

(iv) protein C,

for the preparation of a medicament for promoting wound healing in a subject.

In a fifth aspect, the present invention provides the use of an agent comprising one or more of;

5 (i) an APC,

(ii) a functional fragment of an APC,

(iii) an APC mimetic compound, and

(iv) protein C,

for the preparation of a delivery system, said delivery system being suitable for application to a
10 wound and thereafter promoting wound healing.

Detailed disclosure of the Invention:

The APC and/or protein C utilised in the present invention may be obtained by purification from a suitable source (eg blood taken from humans or other animals) or produced
15 by standard recombinant DNA techniques such as is described in, for example, Maniatis, T. et al., Molecular Cloning: a laboratory manual, Second Edition, Cold Spring Harbor Laboratory Press. Recombinant APC or protein C may incorporate modifications (eg amino acid substitutions, deletions, and additions of heterologous amino acid sequences), which may, for example, enhance biological activity or expression of the respective protein. However,
20 preferably, the present invention utilises human APC and/or protein C. The APC and/or protein C may also be glycosylated by methods well known in the art and which may comprise enzymatic and non-enzymatic means.

Suitable functional fragments of an APC may be produced by cleaving purified natural APC or recombinant APC with well known proteases such as trypsin and the like, or more
25 preferably, by recombinant DNA techniques or peptide/polypeptide synthesis. Such functional fragments may be identified by generating candidate fragments and assessing biological activity by, for example, assaying for activation of MMP-2, promotion of repair of a wounded endothelial monolayer and/or angiogenesis in chicken embryo chorio-alantoic membrane (CAM) in a manner similar to that described in the examples provided herein. Preferably,
30 functional fragments will be of 5 to 100 amino acids in length, more preferably, of 10 to 30 amino acids in length. The functional fragments may be linear or circularised and may include modifications of the amino acid sequence of the native APC sequence from whence they are derived (eg amino acid substitutions, deletions, and additions of heterologous amino acid sequences). The functional fragments may also be glycosylated by methods well known in the
35 art and which may comprise enzymatic and non-enzymatic means.

Suitable APC mimetic compounds (ie compounds which mimic the function of APC) may be designed using any of the methods well known in the art for designing mimetics of

peptides based upon peptide sequences in the absence of secondary and tertiary structural information (28). For example, peptide mimetic compounds may be produced by modifying amino acid side chains to increase the hydrophobicity of defined regions of the peptide (eg substituting hydrogens with methyl groups on aromatic residues of the peptides), substituting amino acid side chains with non-amino acid side chains (eg substituting aromatic residues of the peptides with other aryl groups), and substituting amino- and/or carboxy-termini with various substituents (eg substituting aliphatic groups to increase hydrophobicity).
5 Alternatively, the mimetic compounds may be so-called peptoids (ie non-peptides) which include modification of the peptide backbone (ie by introducing amide bond surrogates by, for example, replacing the nitrogen atoms in the backbone with carbon atoms), or include *N*-substituted glycine residues, one or more D-amino acids (in place of L-amino acid(s)) and/or one or more α -amino acids (in place of β -amino acids or γ -amino acids). Further mimetic compound alternatives include "retro-inverso peptides" where the peptide bonds are reversed and D-amino acids assembled in reverse order to the order of the L-amino acids in the peptide
10 sequence upon which they are based, and other non-peptide frameworks such as steroids, saccharides, benzazepine, 1,3,4-trisubstituted pyrrolidinone, pyridones and pyridopyrazines. Suitable mimetic compounds may also be designed/identified by structural modelling/determination, by screening of natural products, the production of phage display libraries (29), minimised proteins (30), SELEX (Aptamer) selection (31), combinatorial libraries
15 and focussed combinatorial libraries, virtual screening/database searching (32), and rational drug design techniques well known in the art (33).

The present invention is suitable for promoting wound healing generally, but is particularly suitable for application to the promotion of slow-healing wounds, otherwise known as "chronic wounds", "impaired-healing wounds" or "ulcers", and may be of any of the
25 wound types discussed above. However, preferably, the present invention is applied to wounds selected from the group consisting of;

- (i) dermal ulcers such as those associated with pressure, vasculitis, arterial and venous diseases (eg in patients suffering from diabetes, in aged patients, associated with venous insufficiency and cerebrovascular incidents, and resulting from pressure sores or localised areas
30 of tissue damage resulting from direct pressure on the skin or from shearing forces and friction),
- (ii) burns,
- (iii) oral wounds (eg caused by gingivitis),
- (iv) eye wounds (eg corneal wounds resulting from injury, surgery or laser therapy),
- 35 (v) non-cutaneous wounds (eg stomach/oesophageal ulcers, vaginal ulcers and internal injury or surgery (including plastic surgery),
- (vi) ischemia-reperfusion injury (eg resulting from myocardial infarction),

(vii) bone and cartilage damage as occurs in musculoskeletal disorders such as rheumatoid arthritis and osteoarthritis, and

(viii) warfarin-related skin necrosis.

The agent may be one or a mixture of any or all of the group consisting of an APC, a
5 functional fragment of an APC, an APC mimetic compound, and a protein C.

The agent is preferably administered to a subject after a sufficient period of time since wounding has elapsed such that the coagulation/inflammation stages of the wound healing process have substantially concluded. In practice, it is preferable that administration of the agent occur within 1 to 48 hours after wounding, more preferably within 1 to 10 hours after
10 wounding.

The agent may be administered to a subject through oral or systemic routes or by direct application (e.g. topical administration) to the wound as a medicament formulation, or as incorporated into a delivery system (eg gelatin sponge such as Gelfoam, fine gauze, nylon mesh, or an adhesive plastic strips such as a Band-aid™) which is applied to the wound.

15 The effective amount of the agent may be expected to vary depending upon the type, site and seriousness of the wound to be treated. It would be well within the skill of persons skilled in the art to adjust the amount appropriately to obtain optimal results. It is, however, expected that generally the effective amount of the agent will be in the range of 0.01 to 10000 µg per kg of body weight, more preferably between 0.1 and 1000 µg per kg of body weight, and most
20 preferably between about 1 and 200 µg per kg of body weight.

Medicaments according to the present invention preferably include an amount of the agent in the range of 0.01 to 1000 µg per g of medicament, in admixture with a pharmaceutically-acceptable carrier (eg gelatin and/or collagen for cream or gel medicaments; isotonic saline, a phosphate buffered solution or the like for drops; or materials such as starch,
25 gelatin, agar, sugar, carboxymethylcellulose, polyvinylalcohol, magnesium stearate, and sodium alginate for dry powders). For use with non-cutaneous wounds and ischemia-reperfusion injury, the medicaments of the present invention may be formulated for oral or systemic administration.

Medicaments and delivery systems (ie gels, sponges, gauzes and meshes) according to
30 the present invention, may contain one or more other active compounds or substances such as other molecules involved in the protein C pathway (eg protein S, EPCR, factor V/Va or factor VIII/VIIIa); antimicrobial agents such as chlorhexidine, povidine-iodine and ciprofloxacin; anticoagulants such as heparin or antithrombin III; steroids such as dexamethasone; inhibitors of inflammation; cardiovascular drugs such as calcium channel blockers; cytokines/growth
35 factors such as epidermal growth factor; local anaesthetics such as bupivacaine; antitumor drugs such as taxol; polyclonal, monoclonal or chimeric antibodies, or functional derivatives or fragments thereof such as antibodies to regulate cell proliferation.

Further, where the medicaments and delivery systems according to the present invention utilise protein C, the medicaments and delivery systems may also include a suitable amount of an agent for activating the protein C (eg thrombin, kallikrein and/or thrombomodulin).

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

The invention will hereinafter be further described by way of the following non-limiting examples and accompanying figures.

Brief description of accompanying figures:

Figure 1 shows the effect of APC on repair of a "wounded" foreskin endothelial (FSE) cell monolayer in culture. Cell monolayers were scraped with a pipette tip, washed and covered with medium containing various amounts of APC, PMA or no treatment. The cells were incubated at 37 degrees C for 24 hr before being measured for wound closure as described in the example. Results shown represent the number of cells migrating into the wound (mean \pm SD) per selected unit of area of duplicate wounds. Similar results were obtained in two separate experiments.

Figure 2 shows the effect of APC on chicken embryo chorio-allantoic membrane (CAM). Chicken embryos were cracked into a sterile weigh boat on day 1. On day 4, gelatin sponges were treated with 5 μ g APC in phosphate buffered saline (PBS) or PBS alone and then placed on top of the CAM. On day 9, the embryos were fixed in Bouin's fluid, sectioned perpendicular to the top surface of the CAM, stained with Masson's stain and viewed under light microscopy. APC-treated CAM showing complete re-epithelialisation over sponge (thin arrows) (A) and many new blood vessels (thick arrows) and fibroblast infiltration (hatched arrows) (B). In the PBS-treated sponges there is no epithelial regrowth (thick arrows) and negligible invasion of cells into the sponge (C,D).

Figure 3 shows the effect of APC on a rat wound healing model. Sprague-Dawley rats were wounded and then treated with A) saline or B) 20 μ g APC and photographed after 4 days. C) Summary of results using 2 rats (total 8 wounds) each treated with saline or 20 μ g APC. Results shown are mean \pm S.D of wound area (mm²) after 40 hr, 4 days and 7 days.

Figure 4 shows the effect of different doses APC on a rat wound healing model. Normal rats were wounded using an 8mm punch biopsy and immediately treated with 0 μg (Control, 3 rats, 12 wounds), 10 μg (3 rats, 12 wounds), 40 μg (4 rats, 16 wounds), 70 μg (3 rats, 12 wounds) or 100 μg (3 rats, 12 wounds) of APC. Wound size was measured by image analysis after 1, 3, 5, 7 and 9 days and results shown are mean \pm S.D of wound area (mm^2) after 1, 2, 3, 4, 5, 7 and 9 days.

Figure 5 shows the effect of APC on a rat wound healing model. Normal rats were wounded using an 8mm punch biopsy and not treated (Control, 2 rats, 7 wounds) or treated twice with APC (40 μg APC immediately after wounding followed by a second treatment of 40 μg APC after 1 day) (3 rats, 11 wounds). Wound size was measured by image analysis and results shown are mean \pm S.D of wound area (mm^2) after 1, 2, 3, 4, 5, 7 and 9 days

Figure 6 shows the effect of APC on a diabetic rat wound healing model. Streptozotocin-induced diabetic rats were wounded using an 8mm punch biopsy and immediately treated with 20 μg APC (2 rats, 7 wounds) or no test agent (Control; 1 rat, 4 wounds). Wound closure was assessed visually and after 1, 2, 3, 4, 5, 7 and 9 days. There was a significant difference in the rate of wound healing (slope of the regression lines) between the control and APC-treated rats, with the latter healing faster ($p < 0.01$).

Example 1: APC promotion of endothelial wound repair.

Activated protein C (APC) was tested for its ability to promote repair of endothelial wounding using a modification of an *in vitro* assay, as described previously (19). Briefly, confluent microvascular endothelial cells (FSE) from neonatal foreskins were cultured for 5 days in 24-well culture plates in growth medium (Biorich plus 50 $\mu\text{g}/\text{ml}$ heparin, 50 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement and 5% human serum). The endothelial monolayers were wounded by a single stroke across the diameter of the well with a pipette tip. The media and dislodged cells were then aspirated, and the plates rinsed with Hanks buffer. Fresh growth medium was added to the plates along with APC at various concentrations or the potent tumour-promoting angiogenic factor, phorbol myristate acetate (PMA) (10 ng/ml) and the cells were incubated at 37 degrees C. After 24 hr, the width of the wound was visualised microscopically and results at different doses of APC were quantified using image analysis and a dose-response curve was generated (Figure 1). Cells cultured in the presence of 75nM APC achieved almost complete wound closure within 24 hours, and showed more than twice the migratory response than did cells without APC. At 100nM there was no further increase. APC (75nM) had approximately the same activity as PMA.

Example 2: APC promotion of angiogenesis.

In view of the ability of APC to activate gelatinase A and promote endothelial wounding, APC was investigated as to whether it could promote angiogenesis. APC was

added to the chicken embryo chorio-allantoic membrane (CAM) assay using gelatin sponges (Gelfoam). Sponges were cut to approximately 2mm x 2mm. Five µg APC in phosphate buffered saline (PBS) or PBS alone was added to gelatin sponges which were subsequently placed on the 9 day old CAM, as previously described (34). The CAMs were inspected daily and on day 14 were photographed and fixed for histological sectioning. Macroscopically, on day 14, the APC-treated gelatin sponges were surrounded by blood vessels that grew radially inwards towards the sponge in a "spoke-wheel" pattern (data not shown). In contrast, gelatin sponges treated with PBS had no surrounding vascular formation. Histological sections showed that APC-treated sponges were infiltrated with many new blood vessels (angiogenesis) (Figure 2). In addition, there was a large migration of fibroblasts into the APC-treated sponges. There was also marked proliferation of the epithelial layer, with the ectoderm completely growing over the gelatin sponge. Associated with this re-epithelialisation was stratification and involution at the periphery of the sponge. The endoderm also demonstrated stratification with villous formation in some sections and the presence of cells being shed from the villi (not shown). In contrast to the APC-treated sponges, there was little evidence of any re-epithelialisation, endothelial cell or fibroblast infiltration in the PBS control sponges.

Example 3: APC promotion of wound healing.

In view of APC's ability to stimulate endothelial migration and enhance re-epithelialisation, fibroblast invasion and angiogenesis, APC was examined for a capacity to improve wound healing in a rat model. Sprague-Dawley rats were anaesthetised and four full-thickness wounds were excised, using a 8mm punch biopsy, on the back of the rat, exposing the underlying dorsolateral skeletal muscle fascia. Hemostasis was achieved by even compression with sterile gauze. APC was diluted in isotonic, sterile, pyrogen-free saline solution and each excision was treated with a 50 µl topical application of sterile, pyrogen-free saline solution or saline containing 20 µg APC. The wounds were left open with no dressing and rats caged one per cage. Wound closure was assessed visually and after 40hr, 4 days and 7 days. At each timepoint, the wounds were digitally photographed using a Nikon Coolpix 950, with a distance calibration scale in the frame. The area of the wound was calculated by image analysis (Scion Image). After 40 hr there was a marked visual improvement in the wound closure in APC-treated wounds compared to controls. On day 4 image analysis results revealed a significant reduction in wound size of APC-treated wounds compared to controls (Figure 3). This difference was maintained on day 7 (Figure 3c, **p<0.01, ***p<0.001).

Example 4: APC promotion of wound healing.

APC was further examined for a capacity to improve wound healing in a rat model. Sprague-Dawley rats were anaesthetised and four full-thickness wounds were excised, using a

8mm punch biopsy, on the back of the rat, exposing the underlying dorsolateral skeletal muscle fascia. Hemostasis was achieved by even compression with sterile gauze. APC was diluted in isotonic, sterile, pyrogen-free saline solution and each excision was immediately treated with a 50 µl topical application of sterile, pyrogen-free saline solution or saline containing the following: 0 µg APC (Control, 3 rats, 12 wounds), 10 µg APC (3 rats, 12 wounds), 40 µg APC (4 rats, 16 wounds), 70 µg APC (3 rats, 12 wounds) or 100 µg APC (3 rats, 12 wounds). The wounds were left open with no dressing and rats caged one per cage. Wound size was measured by image analysis after 1, 3, 5, 7 and 9 days. At each timepoint, the wounds were digitally photographed using a Nikon Coolpix 995. The area of the wound was calculated by image analysis (Scion Image). Results are shown in Figure 4. After 1 day, there was a significant reduction in the size of the wounds treated with 10 or 40 µg APC. There was no difference between the controls and rats treated with 70 or 100 µg APC. The significant reduction in wound size was most notable with 40 µg APC and seen on days 1, 3, 7 and 9. (** p<0.01, * p<0.05, Student's t-test, using CoStat).

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Example 5: APC promotion of wound healing.

APC was further examined for a capacity to improve wound healing in a rat model. Sprague-Dawley rats were wounded as described in Example 4. APC was diluted in isotonic, sterile, pyrogen-free saline solution and each excision was treated with a 50 µl topical application of sterile, pyrogen-free saline solution or saline containing 40 µg APC. After 48hr, wounds were treated with a second application of 40 µg APC. The wounds were left open with no dressing and rats caged one per cage. Wound closure was assessed visually and after 1, 2, 3, 4, 5, 7 and 9 days. At each timepoint, the wounds were digitally photographed using a Nikon Coolpix 995. The area of the wound was calculated by image analysis (Scion Image). Results are shown in Figure 5. There was a significant difference in the size of the wounds after 1 day, with APC-treated rats healing faster than controls (p<0.01). This difference was also observed at days 2, 3 and 7 (** p<0.01, * p<0.05, Student's t-test, using CoStat).

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Example 6: APC promotion of wound healing in diabetic rats.

APC was examined for a capacity to improve wound healing in a diabetic rat model. The diabetic model was selected because it is a well-described model for slow wound healing (35). Diabetes was induced in Sprague-Dawley rats using the standard procedure of an IP injection of streptozotocin. After 1 week, the blood glucose levels of the rats were >20mM, indicative of diabetes. Diabetic rats were wounded using an 8mm punch biopsy, as described above in Example 4, and immediately treated with 20 µg APC (2 rats, 7 wounds) or no test agent (Control, 1 rat, 4 wounds). The wounds were left open with no dressing and rats caged one per cage. Wound closure was assessed visually and after 1, 2, 3, 4, 5, 7 and 9 days. At each

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timepoint, the wounds were digitally photographed using a Nikon Coolpix 995. The area of the wound was calculated by image analysis (Scion Image). Results are shown in Figure 6. There was a significant difference in the rate of wound healing (slope of the regression lines) between the control and APC-treated rats, with the latter healing faster ($p < 0.01$).

5 **Conclusion:**

The ability of APC to repair endothelial wounding promote re-epithelialisation, fibroblast infiltration and angiogenesis, as well as accelerate wound healing in the rat, indicates that it will be an effective wound healing agent.

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It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A method for promoting wound healing in a subject, said method comprising administering to said subject an effective amount of an agent comprising one or more of;
5 (i) an activated protein C (APC),
(ii) a functional fragment of an APC,
(iii) an APC mimetic compound, and
(iv) protein C,
optionally in admixture with a pharmaceutically-acceptable carrier.
10
2. The method of claim 1, wherein the agent is APC.
3. The method of claim 2, wherein the agent is human APC.
- 15 4. The method of claim 1, wherein the agent is protein C.
5. The method of claim 4, wherein the agent is human protein C.
6. The method of any one of claims 1, 4 and 5, wherein the protein C is administered with
20 an activator for protein C.
7. The method of claim 6, wherein the activator is selected from the group consisting of thrombin, kallikrein and/or thrombomodulin.
- 25 8. The method of claim 1, wherein the agent is a functional fragment of APC.
9. The method of claim 1, wherein the agent is an APC mimetic compound.
10. The method of any one of claims 1 to 9, wherein the agent is administered to said subject
30 within 1 to 10 hours of wounding.
11. The method of any one of claims 1 to 10, wherein the wound for which wound healing is to be promoted is selected from the group consisting of dermal ulcers, burns, oral wounds, eye wounds, non-cutaneous wounds, ischemia-reperfusion injury, bone and cartilage damage and
35 warfarin-related skin necrosis.

12. The method of claim 11, wherein the wound for which wound healing is to be promoted is a dermal ulcer.
13. The method of any one of claims 1 to 12, wherein the agent is administered topically.
- 5 14. The method of any one of claims 1 to 13, wherein the effective amount of the agent is in the range of 0.1 to 1000 μg per kg of body weight.
- 10 15. The method of claim 14, wherein the effective amount of the agent is in the range of 0.1 to 10 μg per kg of body weight.
16. A medicament for promoting wound healing in a subject, said medicament comprising an amount of an agent comprising one or more of;
- 15 (i) an activated protein C (APC),
(ii) a functional fragment of an APC,
(iii) an APC mimetic compound, and
(iv) protein C,
in admixture with a pharmaceutically-acceptable carrier.
- 20 17. The medicament of claim 16, wherein the agent is human APC.
18. The medicament of claim 16, wherein agent is human protein C.
19. The medicament of claim 16 or 18, further comprising an activator for the protein C.
- 25 20. The medicament of claim 19, wherein the activator is selected from the group consisting of thrombin, kallikrein and/or thrombomodulin.
21. The medicament of any one of claims 16 to 20, wherein the medicament is for treatment
30 of a wound selected from the group consisting of dermal ulcers, burns, oral wounds, eye wounds, non-cutaneous wounds, ischemia-reperfusion injury, bone and cartilage damage and warfarin-related skin necrosis.
22. The medicament of claim 21, wherein the medicament is for treatment of a dermal ulcer.
- 35 23. The medicament of any one of claims 16 to 22, wherein the medicament is for topical administration.

24. A delivery system incorporating an amount of an agent comprising one or more of;
- (i) an activated protein C (APC),
 - (ii) a functional fragment of an APC,
 - 5 (iii) an APC mimetic compound, and
 - (iv) protein C,
- said delivery system being suitable for application to a wound and thereafter promoting wound healing.
- 10 25. The delivery system of claim 24, wherein the agent is human APC.
26. The delivery system of claim 24, wherein the agent is human protein C.
27. The delivery system of claim 24 or 26, further comprising an activator for the protein C.
- 15 28. The delivery system of claim 27, wherein the activator is selected from the group consisting of thrombin, kallikrein and/or thrombomodulin.
29. The delivery system of any one of claims 24 to 28, wherein the delivery system is a gel, sponge, gauze or mesh.
- 20 30. The use of an agent comprising one or more of;
- (i) an activated protein C (APC),
 - (ii) a functional fragment of an APC,
 - 25 (iii) an APC mimetic compound, and
 - (iv) protein C,
- for the preparation of a medicament for promoting wound healing in a subject.
31. The use of claim 30, wherein the agent is human APC.
- 30 32. The use of claim 30, wherein the agent is human protein C.
33. The use of claim 32, wherein the medicament further comprises an activator for the protein C.
- 35 34. The use of claim 33, wherein the activator is selected from the group consisting of thrombin, kallikrein and/or thrombomodulin.

35. The use of any one of claims 30 to 34, wherein the medicament is for treatment of a wound selected from the group consisting of dermal ulcers, burns, oral wounds, eye wounds, non-cutaneous wounds, ischemia-reperfusion injury, bone and cartilage damage and warfarin-
5 related skin necrosis.
36. The use of claim 35, wherein the medicament is for treatment of a dermal ulcer.
37. The use of any one of claims 30 to 36, wherein the medicament is for topical
10 administration.
38. The use of an agent comprising one or more of;
(i) an activated protein C (APC),
(ii) a functional fragment of an APC,
15 (iii) an APC mimetic compound, and
(iv) protein C,
for the preparation of a delivery system, said delivery system being suitable for application to a wound and thereafter promoting wound healing.
- 20 39. The use of claim 38, wherein the agent is human APC.
40. The use of claim 38, wherein the agent is human protein C.
41. The use of claim 40, wherein the delivery system further comprises an activator for the
25 protein C.
42. The use of claim 41, wherein the activator is selected from the group consisting of thrombin, kallikrein and/or thrombomodulin.
- 30 43. The use of any one of claims 38 to 42, wherein the delivery system is a gel, sponge, gauze or mesh.

Figure 1

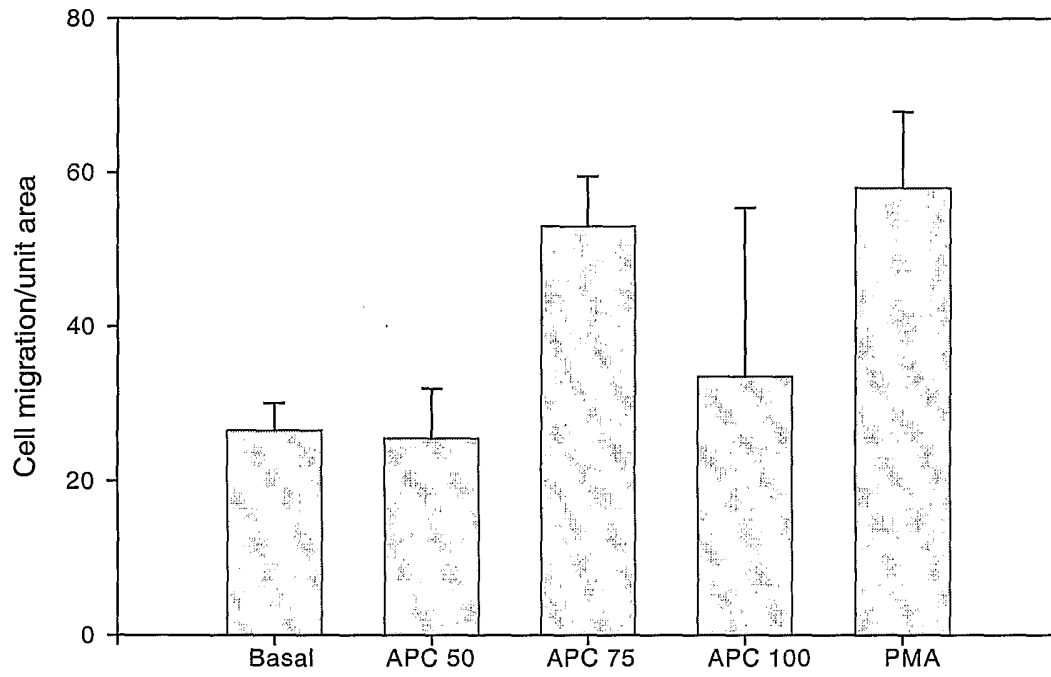


Figure 2

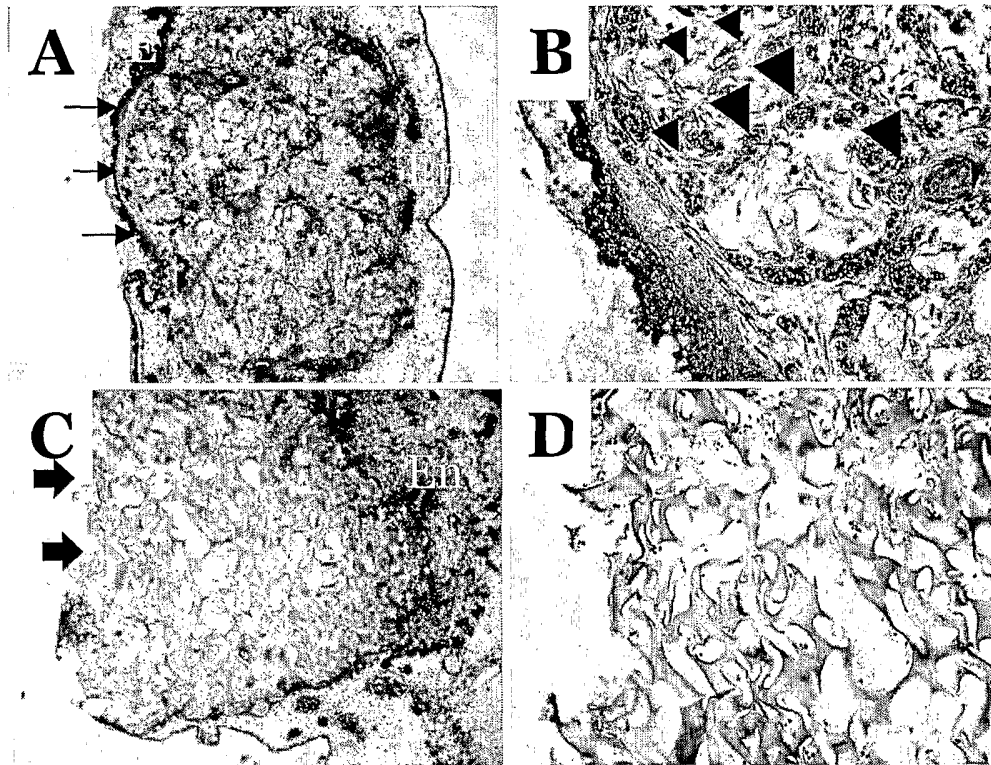


Figure 3

A control

B APC treated

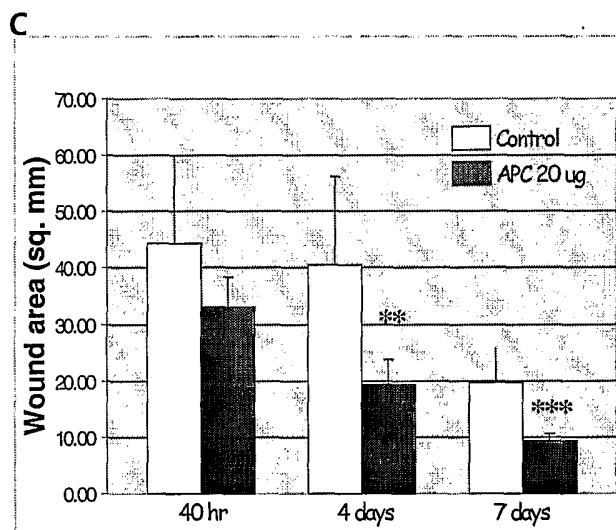
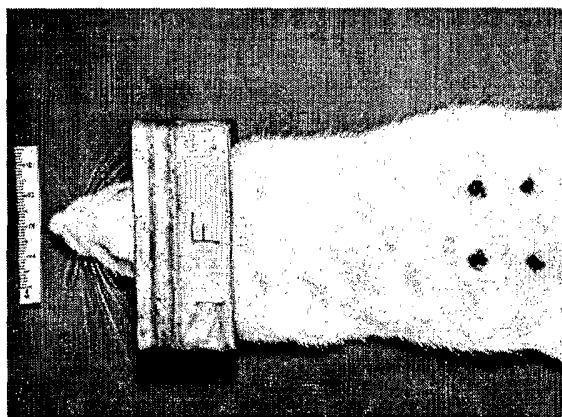
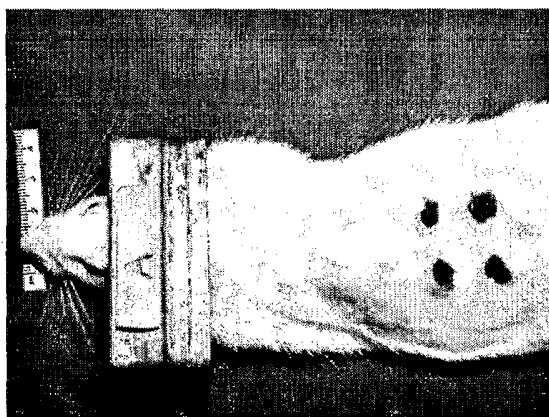


Figure 4

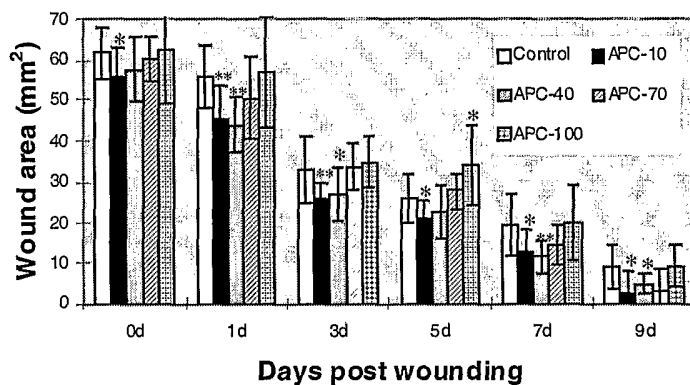


Figure 5

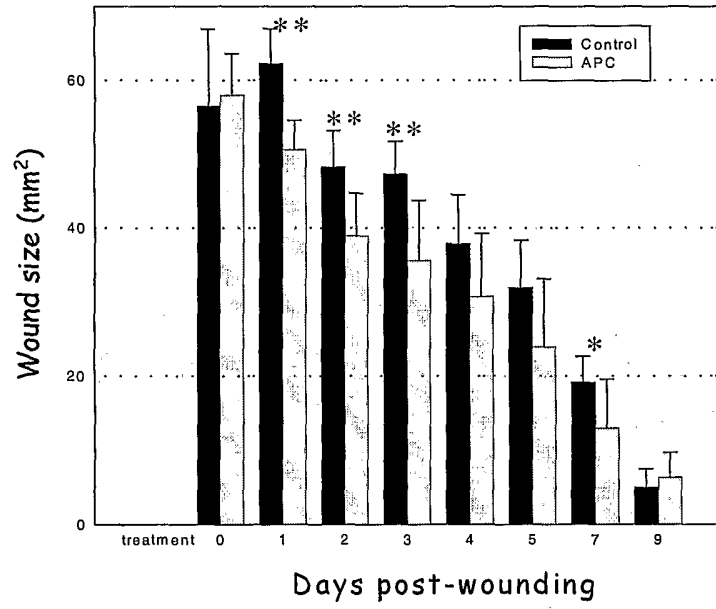
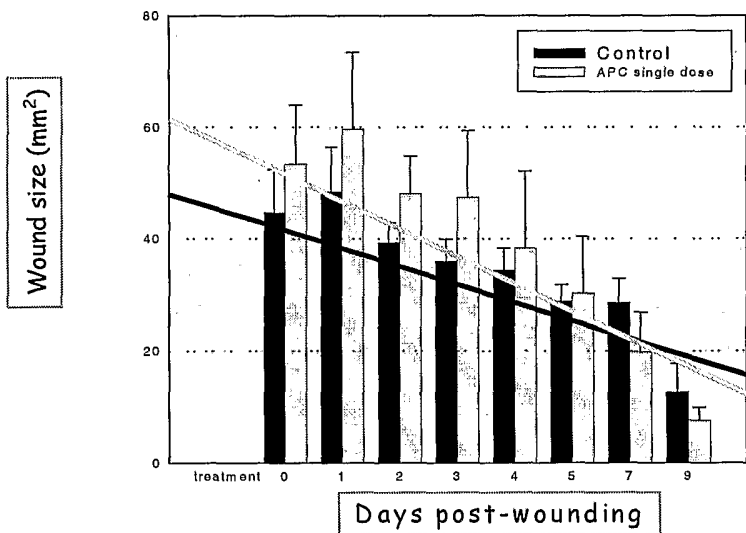


Figure 6



INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00751

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl. ⁷: A61K 038/36, 038/48; A61P 9/10, 17/02, 19/04, 19/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPAT, CAPLUS, PUBMED: Protein C AND (wound OR ulcer OR burn OR ischaemia OR gingivitis OR cornea OR arthritis)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/02172A (Oklahoma Medical Research Foundation) 3 February 1994 See whole document	1, 4-7, 10-11, 13-16, 18-21, 23, 30, 32-35, 37-38, 41-42
X	WO 95/30429A (Board of Trustees of the University of Arkansas) 16 November 1995 See whole document	1, 4-5, 10-11, 13-16, 18, 21, 23, 30, 32, 35, 37, 38, 40
X	US 5,571,786A (EIBL, Johann et al.) 5 November 1996 See whole document	1-7, 11, 14- 21, 30-35

 Further documents are listed in the continuation of Box C See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 July 2002

Date of mailing of the international search report

9 AUG 2002

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00751

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/20293A (Eli Lilly and Company) 29 April 1999 See whole document	1-3, 10-11, 14-17, 21, 30-31, 35
P,X	WO 01/72328A (Eli Lilly and Company) 4 October 2001 See whole document	1-3, 10-11, 14-17, 21, 30-31, 35
X	Hirose, Koji et al. "Activated Protein C Reduces the Ischemia/Reperfusion-Induced Spinal Cord Injury in Rats by Inhibiting Neutrophil Activation" Annals of Surgery Vol.232(2) (August 2002) pages 272-280 See whole document	1-3, 10-11, 14-17, 21, 30-31, 35
X	Taoka, Yuji et al. "Activated Protein C Reduces the Severity of Compression-Induced Spinal Cord Injury in Rats by Inhibiting Activation of Leukocytes" The Journal of Neuroscience Vol.18(4) (15 February 1998) pages 1393-1398 See whole document	1-3, 10-11, 14-17, 21, 30-31, 35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU02/00751

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member			
WO 9402172	EP	651655		
WO 9530429	AU	24673/95		
US 5571786	AT	1697/90	AT	203/92
	CA	2041380	AT	204/92
	EP	533209	CS	9101213
	HU	60147	EP	533210
	CA	2209931	EP	533210
WO 9920293	AU	64741/98	EP	533210
	BR	9812965	JP	4230633
	EP	913156	EP	804228
	NO	20002005	WO	9622108
	PL	340096	AU	98040/98
	US	6156734	BR	9808063
	US	2001036456	CA	2306983
	HU	200001237	HU	200100025
WO 200172328	AU	200145319	NZ	337291
			US	6008199
			US	6268337
			US	6268344
			US	6268344
			WO	9842358
			ZA	9809385
			ZA	9802489

END OF ANNEX