Activated protein C (APC) as a novel agent to promote wound healing

Jackson CJ • Xue M

Abstract

Activated protein C (APC) is a serine protease that plays a central role in physiological anticoagulation and has, more recently, been shown to be a potent anti-inflammatory mediator. Recent work in our lab shows that APC upregulates expression and activation of matrix metalloproteinase-2 (MMP-2), an enzyme that plays a prominent role during angiogenesis in cultured human skin fibroblasts (HF), endothelial cells and keratinocytes (HK). Furthermore, APC promotes the migration and proliferation of these cells *in vitro*. In a full-thickness rat skin healing model, APC enhances wound healing compared to saline control.

In summary, our results demonstrate that APC promotes cutaneous wound healing at least partly by upregulating MMP-2 activity, increasing angiogenesis, promoting re-epithelialisation and dampening inflammation. These unique properties of APC make it an attractive therapeutic agent to promote the healing of chronic wounds.

Jackson CJ & Xue M. Activated protein C (APC) as a novel agent to promote wound healing. Primary Intention 2006; 14(1):31-34.

Introduction

Healing of cutaneous wounds is a complex, well-orchestrated physiological event involving a series of dynamic events, including formation of fibrin clot, inflammatory response, granulation tissue formation (incorporating re-epithelialisation and angiogenesis) and matrix formation and remodelling [1]. Although some of these stages overlap, for normal wound healing to proceed it is important that the temporal sequence of these events is maintained. That is, the early events of coagulation and inflammation occur quickly and then cease in order to allow the subsequent reparative processes to occur. Conversely, a chronic wound occurs when this normal integrated sequential process of healing is disrupted, such as prolonged inflammation [2, 3], imbalanced enzyme activity, or reduction or absence of angiogenesis [4] which results in prolonged or incomplete healing.

Christopher John Jackson*
Meilang Xue
Sutton Arthritis Research Laboratory
University of Sydney
Royal North Shore Hospital
St Leonards NSW, 2065
Tel: (02) 9926 6043
Fax: (02) 9926 6269
E-mail: cjackson@med.usyd.edu.au

* Corresponding author

APC as a anti-inflammatory mediator

This study shows how APC damps inflammation, increases matrix metalloproteinase-2 (MMP-2) activity, stimulates angiogenesis, stimulates the proliferation and migration of human skin keratinocytes (HK) and promotes rat wound healing.

APC damps inflammation

Protein C is a plasma serine protease that plays a key role in maintaining normal homeostasis. The thrombin-activated form of APC acts as a feedback inhibitor of the coagulation cascade and has anti-thrombotic activity in numerous model systems [5, 6]. Recently, APC has been shown both *in vivo* and *in vitro* to have significant anti-inflammatory properties associated with a decrease in proinflammatory cytokines and a reduction of leukocyte recruitment [7, 8]. *In vitro*, APC suppresses nuclear factor (NF) β pathway in monocytes [9, 10], endothelial cells [11] and keratinocytes [12]. APC also inhibits LPS-induced TNF-β expression in a monocytic cell line [9] and inhibits endothelial cell apoptosis [13]. The importance of APC as an anti-inflammatory agent is reflected by the finding that it is effective in treating patients with severe sepsis [14].

APC increases MMP-2 activity

MMP-2 is a member of zinc-dependent endopeptidases that play a vital role in the tissue repair process by remodelling the extracellular matrix (ECM) [15]. Tight MMP regulation at the level of synthesis, secretion, activation and inhibition is critical to maintain normal function.

The regulation of MMP-2 is different to the other 24 known human MMPs. MMP-2 is not responsive to inflammatory cytokines but constitutively secreted as a latent enzyme by most cell types. In addition, MMP-2 is resistant to direct cleavage by enzymes that cleave other MMPs and can be activated by a unique mechanism on the cell surface through involving membrane type (MT)-MMPs and tissue inhibitor of MMP-2 (TIMP-2) [16, 17].

We have shown that APC can rapidly, directly, efficiently and specifically activate MMP-2 [18] (Figure 1). Furthermore, APC can upregulate gene and protein expression of MMP-2 in human endothelial cells, human skin fibroblasts (HF) and HK (Figure 1). By degrading the collagens present in the basement membrane, MMP-2 plays an important role in cellular invasive processes that occur during angiogenesis and re-epithelialisation.

APC stimulates angiogenesis

APC dose-dependently upregulates the gene and protein expression of other angiogenic factors in cultured human cells. In human umbilical vein endothelial cells (HUVE) and HK, APC stimulates interleukin (IL)-6 and IL-8 expression [12, 19]. Vascular endothelial growth factor (VEGF), the most potent angiogenic mediator known, is markedly enhanced in HK and HF by APC [20] and HF increase their expression of monocyte chemoattractant protein-1 (MCP-1) in response to APC.

To determine whether APC stimulates angiogenesis, we added APC- or saline (control)-treated gelatin sponges to the chick embryo chorio-allantoic membrane (CAM). After 5 days there was a massive formation of fine capillary blood vessels invading the APC-treated gelatin sponges which were not present in the control (Figure 2), indicating that APC stimulates angiogenesis [20]. Uchiba *et al.* [21] have also recently shown that APC induces angiogenesis in the rabbit corneal assay and provided evidence that mitogen-activated protein (MAP) kinase activation was involved as endothelial cell proliferation and was inhibited by MAP kinase inhibition.

APC stimulates the proliferation and migration of HK

In addition to promoting angiogenesis in the CAM assay, APC stimulated ectoderm epithelial cells to grow across the top of the gelatin sponge (Figure 2). This process mimics the regrowth of skin epithelial cells (HK) during the healing of cutaneous wounds. HK are the major cell type of the epidermis and play a fundamental role in normal skin metabolism and cutaneous wound healing. We investigated the regulatory role of APC on the function of human primary cultured HK. In an *in vitro* wounding assay, APC accelerated wound closure which was jointly due to increased cell proliferation and migration [12]. APC also attenuated calcium- and LPS-induced HK apoptosis [12].

The signalling mechanisms of APC's stimulation of HK function are unknown and, to date, no keratinocyte receptor for APC has been identified. Using RT-PCR, Western blotting and immunohistochemistry, we have recently shown that cultured HK express endothelial protein C receptor (EPCR), the major receptor of protein C pathway [22].

EPCR was also strongly expressed by epidermal layers of neonatal foreskin using immunohistochemistry (Figure 3). In

Figure 1. APC upregulates the expression and activation of MMP-2 in cultured cells. Human keratinocytes (HK), fibroblasts (HF) and umbilical vein endothelial cells (HUVE) were treated with 20μg/ml APC for 24 hr. Zymography was used to detect the latent (pro-MMP-2) and active (MMP-2) forms of MMP-2 in culture supernatants.

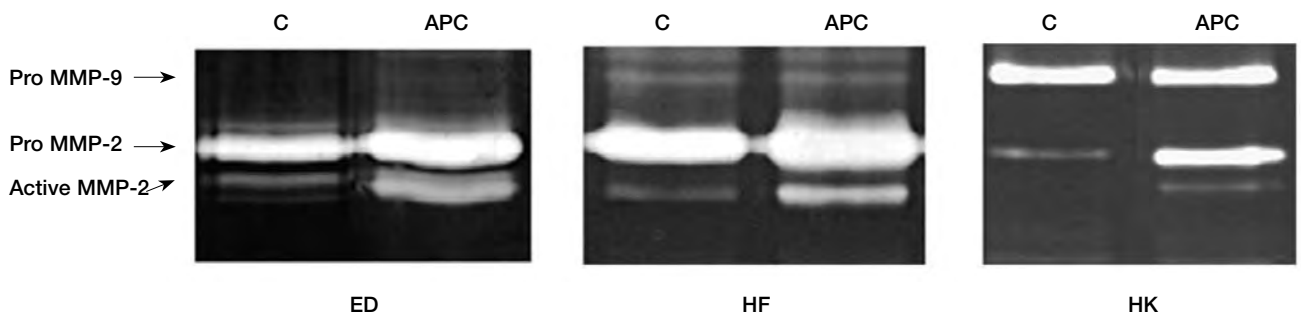


Figure 2. APC stimulates angiogenesis and re-epithelialisation in the CAM assay.
Gelatin sponges (bordered by small arrows) treated with PBS or 10µg APC were removed from the CAM after 5 days and, together with the surrounding area of membrane, were fixed, sectioned and stained with Masson's trichrome stain. Saline-treated (control) sponges stained orange with little evidence of cellular infiltrate. APC-treated sponges were infiltrated by many new blood vessels and showed marked proliferation of the ectodermal epithelial layer (large arrows).

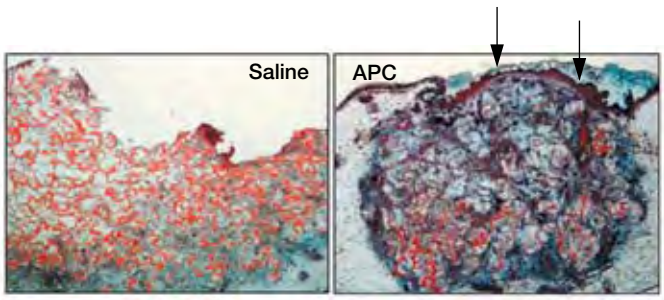
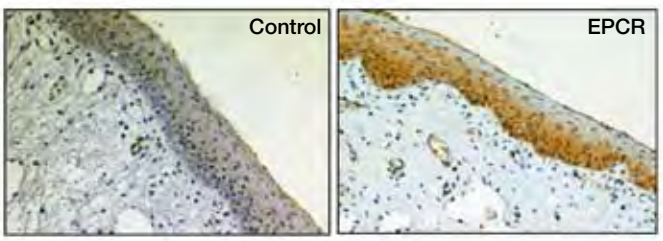


Figure 3. EPCR expression in human foreskin.
Neonatal foreskin sections were processed using immunohistochemistry for expression of EPCR. The sub-epidermal layers and dermal vessels stained positive for EPCR.



cultured HK, EPCR expression was upregulated by the addition of APC and inhibited by tumour necrosis factor-α [22]. RCR252, a monoclonal antibody which blocks APC binding to EPCR, abolished proliferation of HK and induction of MMP-2 by APC [22]. Similar effects were observed with a blocking antibody to PAR-1, indicating that APC's stimulation of HK's function occurs via EPCR and PAR-1 [22]. These results demonstrate a central role for APC in promoting re-epithelialisation by stimulating proliferation and migration, preventing apoptosis and increasing MMP-2 activity in cultured HK (Figure 4).

APC promotes rat wound healing

In a full-thickness rat skin healing model, a single topical application of APC enhanced wound healing compared to saline control. No adverse effects, including toxicity or excessive bleeding, were observed in response to topical APC administration. Wounds treated with 20-40µg APC showed accelerated healing after 40 hours, with wound size noticeably reduced and the surface of the wound less inflamed than the control. This improvement was maintained until wound healing was complete at Day 15 [20]. APC-treated wounds had more blood vessels on Day 7 and a lower infiltration of neutrophils at Days 4 and 7. The broad spectrum MMP inhibitor, GM6001, prevented APC's ability to promote wound healing, suggesting that MMPs were necessary for healing [20]. Thus, APC promotes cutaneous wound healing in the rat via a complex mechanism involving stimulation of angiogenesis and inhibition of inflammation.

Conclusion

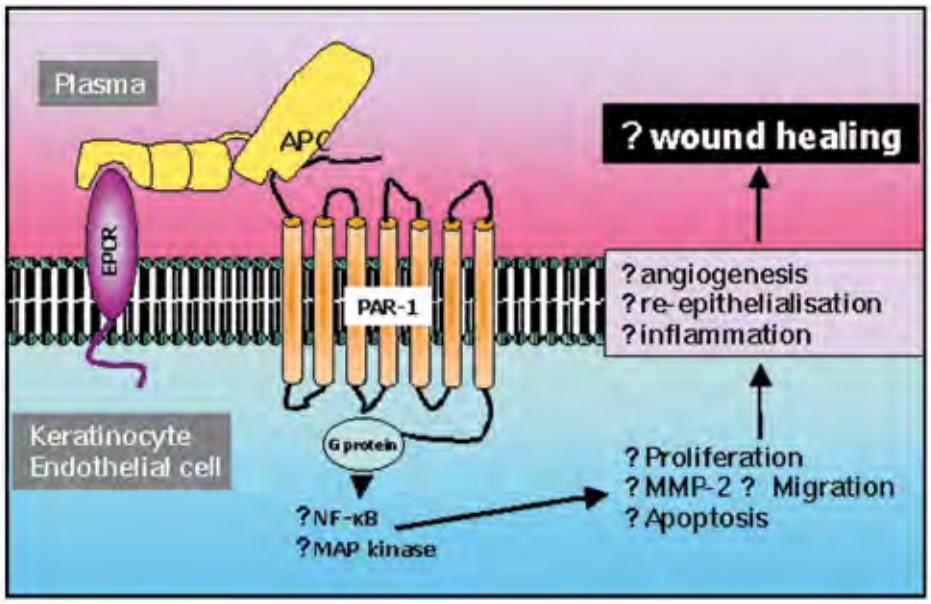
The actions of APC in wound healing are summarised in Figure 4. APC can bind to EPCR on the surface of keratinocytes and endothelial cells and cleave PAR-1 to induce signal transduction via MAP kinase and inhibition of NF-β. This signalling causes increased proliferation, MMP-2 activation and subsequent migration and prevention of apoptosis, leading to stimulation of angiogenesis and re-epithelialisation and inhibition of inflammation to promote wound healing.

These findings highlight an interesting new role for a protein that has classically been defined as an anti-coagulant and, more recently, as an anti-inflammatory mediator. Thus, APC has unique properties which make it an attractive therapeutic agent to promote the healing of chronic wounds, particularly those that become locked in the inflammatory phase of healing and cannot progress to form granulation tissue.

References

1. Nwomeh BC, Yager DR & Cohen IK. Physiology of the chronic wound. Clin Plast Surg 1998; **25(3):**341-56.
2. Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD & Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. J Invest Dermatol 1998; **111(5):**850-7.
3. Trengove NJ, Stacey MC, MacAuley S, Bennett N, Gibson J, Burslem F, Murphy G & Schultz G. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen 1999; **7(6):**442-52.
4. Singer AJ & Clark RA. Cutaneous wound healing. N Engl J Med 1999; **341(10):**738-46.
5. Esmon NL, Safa O, Smirnov MD & Esmon CT. Anti-phospholipid antibodies and the protein C pathway. J Autoimmun 2000; **15(2):**221-5.
6. Esmon CT. Role of coagulation inhibitors in inflammation. Thromb Haemost 2001; **86(1):**51-6.
7. Murakami K, Okajima K, Uchiba M, Johno M, Nakagaki T, Okabe H & Takatsuki K. Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production. Am J Physiol 1997; **272(2 Pt 1):**L197-L202.
8. Uchiba M, Okajima K, Murakami K, Johno M, Okabe H & Takatsuki K. Effect of human urinary thrombomodulin on endotoxin-induced intravascular coagulation and pulmonary vascular injury in rats. Am J

Figure 4. Mechanism of action of APC as a promoter of wound healing.
In keratinocytes and endothelial cells, APC can bind to EPCR and cleave PAR-1 to inhibit NF-β and regulate MAP kinase. This signalling causes increased proliferation, MMP-2 activation and subsequent migration and prevention of apoptosis. These events result in stimulation of angiogenesis and re-epithelialisation and inhibition of inflammation, leading to promotion of wound healing.



Hematol 1997; **54(2):**118-23.

9. Joyce DE & Grinnell BW. Recombinant human activated protein C attenuates the inflammatory response in endothelium and monocytes by modulating nuclear factor-kappaB. Crit Care Med 2002; **30(5 Suppl):**S288-S293.
10. White B, Schmidt M, Murphy C, Livingstone W, O'Toole D, Lawler M, O'Neill L, Kelleher D, Schwarz HP & Smith OP. Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor kappaB (NF-kappaB) and tumour necrosis factor alpha (TNF-alpha) production in the THP-1 monocytic cell line. Br J Haematol 2000; **110(1):**130-4.
11. Joyce DE, Gelbert L, Ciaccia A, DeHoff B & Grinnell BW. Gene expression profile of antithrombotic protein C defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 2001; 276(14):11199-11203.
12. Xue M, Thompson P, Kelso I, Jackson C. Activated protein C stimulates proliferation, migration and wound closure, inhibits apoptosis and upregulates MMP-2 activity in cultured human keratinocytes. Exp Cell Res 2004; **299(1):**119-27.
13. Cheng XW, Kuzuya M, Kanda S, Maeda K, Sasaki T, Wang QL, Tamaya-Mori N, Shibata T & Iguchi A. Epigallocatechin-3-gallate binding to MMP-2 inhibits gelatinolytic activity without influencing the attachment to extracellular matrix proteins but enhances MMP-2 binding to TIMP-2. Arch Biochem Biophys 2003; **415(1):**126-32.
14. Griffin JH, Zlokovic B & Fernandez JA. Activated protein C: potential therapy for severe sepsis, thrombosis and stroke. Semin Hematol 2002; **39(3):**197-205.
15. Ravanti L & Kahari V. Matrix metalloproteinases in wound repair (Review). Int J Mol Med 2000; **6(4):**391-407.
16. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA & Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J Biol Chem 1995; **270(10):**5331-8.
17. Visse R & Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003; **92(8):**827-39.
18. Nguyen M, Arkell J & Jackson CJ. Activated protein C directly activates human endothelial gelatinase A. J Biol Chem 2000; **275(13):**9095-8.
19. Hooper WC, Phillips DJ, Renshaw MA, Evatt BL & Benson JM. The up-regulation of IL-6 and IL-8 in human endothelial cells by activated protein C. J Immun 1998; **161(5):**2567-73.
20. Jackson CJ, Xue M, Thompson P, Davey R, Whitmont K, Smith S, Buisson-Legendre N, Sztynda T, Jackson L, Cooper A, Sambrook P & March L. Activated protein C prevents inflammation yet stimulates angiogenesis to promote cutaneous wound healing. Wound Rep Regen 2005; **13(3):**284-94.
21. Uchiba M, Okajima K, Oike Y, Ito Y, Fukudome K, Isobe H & Suda T. Activated protein C induces endothelial cell proliferation by mitogen-activated protein kinase activation *in vitro* and angiogenesis *in vivo*. Circ Res 2004; **95(1):**34-41.
22. Xue M, Campbell D, Sambrook P, Fukudom K & Jackson C. Endothelial protein C receptor and protease activated receptor-1 mediate induction of a wound healing phenotype in human keratinocytes by activated protein C. J Invest Dermatol. In press.