Activated protein C stimulates proliferation, migration and wound closure, inhibits apoptosis and upregulates MMP-2 activity in cultured human keratinocytes

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Abstract

Activated protein C (APC) is a physiological serine protease that regulates blood clotting and inflammation. Keratinocytes are a major cell type of human skin and play a fundamental role in normal skin metabolism and cutaneous wound healing. In this study, we investigated the regulatory role of APC on the function of human primary cultured keratinocytes. In an in vitro wounding assay, APC accelerated wound closure which was due jointly to increased cell proliferation and migration. APC attenuated calcium-induced cell death via prevention of cell apoptosis, as indicated by a decrease in both active caspase-3 and morphologically apoptotic cells. APC dramatically enhanced the expression and activation of MMP-2 by keratinocytes, whilst having no effect on MMP-9. GM6001, a broad spectrum MMP inhibitor, abolished cell migration in a dose-dependent manner and delayed in vitro wound healing. APC also significantly increased the production of IL-6 and IL-8 and suppressed calcium- and LPS-stimulated NF-κB activity. These results demonstrate a central role for APC in promoting cell proliferation and migration, preventing apoptosis and increasing MMP-2 activity in cultured keratinocytes. This regulatory activity implicates APC as having potential to promote re-epithelialisation during wound healing.

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Introduction

Human protein C is a plasma serine protease that plays a key role in maintaining normal haemostasis. The thrombin-activated form of protein C (APC) acts as a feedback inhibitor of the coagulation cascade and has anti-thrombotic activity in numerous model systems [1,2]. 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. For example, APC prevents lipopolysaccharide (LPS)-induced pulmonary vascular injury and protects against ischaemia–reperfusion-induced renal injury by inhibiting the accumulation and activation of leukocytes [3,4]. In vitro, APC suppresses the nuclear factor (NF)-κB pathway in both human monocytes [5,6] and endothelial cells [7]. APC also inhibits LPS-induced TNF-α expression in a monocytic cell line [6] and inhibits endothelial cell apoptosis [8]. Our recent findings have shown that APC can activate endothelial matrix metalloproteinase (MMP)-2 [9], a member of the MMP family of zinc-dependent endopeptidases that plays a vital role in the tissue repair process by remodeling the extracellular matrix (ECM) [10].

Keratinocytes are the predominant cell type in human skin. Within the epidermis, keratinocytes exist at various stages of differentiation corresponding to different epidermal layers [11,12], in which dividing basal cells withdraw from the cell cycle and progressively differentiate as they are displaced toward the skin surface. Keratinocytes play a fundamental role in skin metabolism and in wound closure, by proliferating and migrating to compensate for superficial cell loss or to cover the exposed connective tissue, and by secreting various mediators including cytokines, chemokines and MMPs. In this study, using primary cultured cells, we have shown that APC regulates proliferation, migration, apoptosis, NF-κB activity and the expression of IL-6, IL-8 and MMP-2 in human keratinocytes.
Methods

Culture and experimental treatment of keratinocytes

Normal keratinocytes were isolated from neonatal foreskin in accordance with local ethics approval and cultured as described previously [13] using keratinocyte serum-free medium (K-SFM, Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with gentamycin (5 μg/ml) plus recombinant epidermal growth factor (EGF) and bovine pituitary extract. When greater than 70% confluent, primary cultured cells were trypsinised and seeded into either 24-well culture plates at 5 × 10^5 cells per well, 96-well plates at 1 × 10^5 cells per well, or 8-well permanox™ slides (Nalge Nunc International Corp., Naperville, IL, USA) and incubated for 12 h to allow adhesion. Adherent cells were assayed as described below in response to the following test agents alone or in combination: recombinant human APC (Eli Lilly, Indiana, USA), Ca^{2+} (CaCl_2), the MMP inhibitor, GM6001 (Chemicon Int., Temecula, CA, USA) or recombinant human EGF (Gibco).

Cell proliferation

To test the ability of APC to stimulate keratinocyte proliferation, 10^5 cells/well were seeded into a 96-well plate in quadruplicate, treated with various concentrations of APC and incubated at 37 °C for 72 h. At the end of experiment, each well was loaded with 5 μl of freshly prepared and filtered MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide, 5 mg/ml in PBS, Sigma) and the mixture was incubated for a further 3 h. The MTT solution was carefully decanted off and formazan in the cells was dissolved with 100 μl of dimethylsulphoxide in each well. The absorbance was measured at a wavelength of 570 nm with a 96-well plate ELISA reader. All MTT assays were conducted in triplicate. The number of keratinocytes at the end of each treatment was also ascertained by direct cell counting.

In vitro migration assay

Cells were seeded into 24-well plates and cultured to subconfluence. Cell monolayers were then scratched with a 1000-μl blue plastic pipette tip (Greiner Bio-one, Greiner International, Longwood, FL, USA), creating a cell-free area (“wound”) approximately 2 mm in width. ‘Wounded’ monolayers were washed twice with K-SFM to remove loose cell debris, and a defined area of the wound was photographed under phase-contrast microscopy. To remove the effect of cell proliferation, cells were pretreated with mitomycin C (10 μg/ml, Sigma, St. Louis, MO, USA), which was applied to the cells 2 h before wounding and removed with three PBS washes. This treatment inhibits the proliferation of cells without killing them [14]. Cultures were then treated with test agents: 1, 5, 10, or 20 μg/ml APC; 1, 5, or 25 μg/ml GM6001; or APC (5 μg/ml) plus GM6001 (1, 5, or 25 μg/ml) for 24 h. EGF (50 ng/ml) was included as a positive control. Cell migration was determined after 24 h by counting the cells that had moved out of the initial area, and the percentage of cell migration calculated as [number of migrated cells following APC treatment / number of migrated cells in control condition] × 100.

In vitro wounding assay

Keratinocytes were grown to confluence in 24-well plates, after which the medium was replaced with K-SFM lacking EGF, and the cells were incubated for a further 24 h. Cell monolayers were then wounded and treated with test agents as described above. A defined area of the wound was photographed under phase-contrast microscopy before treatment. The pre-determined wound area was re-photographed, and wound area remaining determined by image analysis, after 24 h. Wound closure rate was determined using the initial and final wound areas during the wounding experiments, with the percentage wound closure calculated as [(initial − final)/initial] × 100.

RNA extraction and reverse-transcriptase (RT)-PCR

Total RNA was harvested from keratinocytes using TRI Reagent (Sigma). Single-stranded cDNAs were synthesised from total RNA using AMV reverse transcriptase (RT) and Oligo (dT)15 as primer (Promega Corp., Madison, WI, USA). RT products were amplified by PCR to detect gene expression of MMP-2 and MMP-9. β-actin was incorporated as an internal standard. Specific primer sequences are listed in Table 1. For MMP-2 and MMP-9, the amplification reaction was performed in a total volume of 50 μl of 10 mM Tris–HCl pH 8.8, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100. The reaction was run for 30 cycles of 1 min at 95 °C, 1 min annealing at 62 °C and 1 min extension at 72 °C, with 10 min extra extension for the final cycle. For IL-6, IL-8 and β-actin, the amplification reaction was performed in a total volume of 50 μl consisting of 2 μl cDNA, 20 mM Tris–HCl (pH 8.55), 2.5 mM MgCl2, 1 μg/ml BSA, 200 μM

| Table 1: PCR primers for human IL-6, IL-8, MMP-2, MMP-9 and β-actin |
|-----------------|-------------------|
| **PCR product** | **Primer sequences** |
| IL-6 (234 bp)   | Sense 5’ CTCATTGACAAGCGCCTTC-3’  |
|                 | Antisense 5’ GGCAGCTCTGTGCTGATC-3’  |
| IL-8 (263 bp)   | Sense 5’ CCTGGAAGCTCTCTGTTTT-3’  |
|                 | Antisense 5’ CGACGCACTTTCCTCCAAAATT-3’  |
| MMP-2 (390 bp)  | Sense 5’ GGGGCCTCTTGCTGCTGATC-3’  |
|                 | Antisense 5’ TCACAGCTCGGCAA-3’  |
| MMP-9 (406 bp)  | Sense 5’ GGGGCCTCTACGGCCCAACT-3’  |
|                 | Antisense 5’ GAGAATCGCCAGTACGTTT-3’  |
| β-actin (738 bp)| Sense 5’ GTGGCTATCCAGGCTGTTG-3’  |
|                 | Antisense 5’ CATAGTCGGCCTGAGG-3’  |
each dNTPs, 50 pmol each of primers and 1.5U Taq DNA polymerase. The reactions were heated at 94°C for 2 min, then subjected to 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min for 72°C. The final elongation step was performed at 72°C for 10 min. PCR products were separated on a 2% agarose gel and semi-quantified by comparing the intensity of the band of interest to that of an internal standard (β-actin) using image analysis.

Zymography

MMP-2 and MMP-9 protein secretion and activation were detected by gelatin zymography under non-reducing conditions as described previously [15]. Samples (cell culture supernatants, 10 μg protein per lane) were loaded onto a 10% SDS gel containing 0.5 mg/ml gelatin. Gelatinolytic enzymes appeared as clear bands against the blue background of the stained gel. Relative levels of MMP-2 and MMP-9 were semi-quantified using Gel-Pro Analyzer (Media Cybernetics).

Evaluation of apoptosis

Confluent cells grown on 8-well permanox™ slides were treated with 20 μg/ml APC, 3 mM Ca2+, or 20 μg/ml APC plus 3 mM Ca2+. After incubation at 37°C for 24 h, cells were fixed with freshly prepared, ice-cold 4% paraformaldehyde and air-dried. Slides were then stained with haematoxylin before examination by light microscopy. Cells were classified as apoptotic according to the criteria established by Ketabchi et al. [16]. The total number of cells and the number of apoptotic cells in six randomly selected high-power fields were counted.

Electrophoretic mobility shift assay

Keratinocytes were treated with 20 μg/ml APC, 3 mM Ca2+, 100 ng/ml lipopolysaccharide (LPS), or 20 μg/ml APC plus either 3 mM Ca2+ or 100 ng/ml LPS for 4 h and then cells were harvested. Nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer’s instructions. Equal amounts of nuclear extracts were subjected to a test for NF-κB protein-DNA binding using LightShift chemiluminescent EMSA kit (Pierce Chemical Co.) with a biotin end-labeled NF-κB oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') (Invitrogen Life Technology). Briefly, nuclear extracts were incubated with biotin end-labeled NF-κB oligonucleotide for 20 min at room temperature to allow DNA-protein binding. The DNA/protein complexes were then resolved by 6% native polyacrylamide gel electrophoresis and transferred to a Hybond-N+ membrane (Amersham and Pharmacia Biotech). The biotin end-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate.

Immunohistochemical staining

Cultured keratinocytes in 8-well permanox slides were fixed with 4% paraformaldehyde. Slides were then incubated with anti-active caspase-3 (0.25 μg/ml, R&D Systems) overnight at 4°C. Mouse IgG was used as a negative control. Cells were stained with DAKO LSAB+ Systems stain kit (DAKO Corporation, Carpenteria, CA, USA) and counterstained with Haematoxylin and Scotts Bluing Solution.

Statistical analysis

Significance was determined using two-way ANOVA analysis and an unpaired Student's t test. P values less than 0.05 were considered statistically significant.

Results

APC stimulates keratinocyte proliferation, migration and wound closure

When added to keratinocytes at concentrations of 1, 5, 10 and 20 μg/ml for 72 h, APC accelerated cell proliferation in a dose-dependent manner (Fig. 1A). At the highest dose tested, APC enhanced the proliferation rate by approximately 33%. Cell migration was measured by direct counting of cells which had migrated out of initial areas after incubation for 24 h (Fig. 1B). APC stimulated cell migration when used at the lower doses tested (1 and 5 μg/ml) (P < 0.05). At 5 μg/ml, the stimulation of cell migration by APC was equivalent to that of 50 ng/ml EGF. Interestingly, the APC dose–response curve was bell-shaped and when used at the highest concentration tested, 20 μg/ml, cell migration was inhibited relative to the control (P < 0.05).

We further examined whether APC stimulates wound closure in vitro. Scratch ‘wounded’ monolayers of human keratinocytes treated with APC at concentrations of 1, 5 and 10 μg/ml for 24 h displayed significant increases (P < 0.05) in wound closure compared to the control (Fig. 1C). Similar to the results for the migration assay, wound closure was abrogated when APC was used at 20 μg/ml and the wound closure stimulated by 1 μg/ml APC was equivalent to that stimulated by 50 ng/ml EGF.

APC selectively upregulates the expression and activation of MMP-2

MMPs, particularly MMP-2 and MMP-9, are extensively involved in keratinocyte migration and wound closure [17,18]. In this study, we analysed gene and protein expression of these two MMPs following APC treatment. Under basal conditions, cultured keratinocytes constitutively expressed both MMP-2 and MMP-9 mRNA and protein, as detected by RT-PCR and zymography, respectively (Fig.
APC upregulated MMP-2, but not MMP-9, in a dose-dependent manner at both the gene and protein level (dose–response data not shown). At 20 μg/ml, APC increased both gene (\(P < 0.05\), Figs. 2A and B) and protein expression (\(P < 0.01\), Figs. 2C and D) of MMP-2. In addition, zymography revealed that APC treatment induced activated MMP-2, which was not evident in the control cells. Addition of APC to Ca\(^{2+}\)-treated cells also enhanced the production and activation of MMP-2 (\(P < 0.01\)). No difference from basal MMP-9 expression was observed following APC treatment in the presence or absence of calcium (Fig. 2).

**APC upregulates the expression of IL-6 and IL-8**

IL-6 and IL-8 have been reported previously to stimulate the proliferation of keratinocytes [19,20]. We examined gene and protein expression of IL-6 and IL-8 in human keratinocytes by RT-PCR and ELISA, respectively. Results were shown in Fig. 3. Keratinocytes constitutively expressed low levels of IL-6 and IL-8 mRNA and protein. When stimulated by APC for 24 h, both gene and protein expression of these two cytokines were markedly upregulated, approximately 20-fold for IL-6 and 6-fold for IL-8 protein compared to the control.

**APC’s effect on cell migration and wound closure is MMP-dependent**

To elucidate whether APC’s stimulation of migration and wound closure was due to MMP activity, we tested the effect of the broad spectrum MMP inhibitor, GM6001. Under basal conditions, this inhibitor prevented both cell migration and wound closure in a dose-dependent manner, indicating that MMPs are responsible for the basal migration of these cells (Fig. 4). The addition of GM6001 to APC-stimulated cells also reduced migration and wound closure in a dose-dependent manner. Greater than 90% inhibition of both parameters occurred using the highest dose of inhibitor (25 μg/ml) (Fig. 4), suggesting that APC-stimulated migration and wound closure is dependent on MMPs.

**APC attenuates cell death induced by calcium via prevention of apoptosis**

To further explore the mechanisms involving APC’s role in the acceleration of wound healing, we examined the effect of APC on the cell death and apoptosis induced by calcium. Growth of cultured keratinocytes in K-SFM containing low levels of calcium resembles that of basal keratinocytes in situ and increasing the calcium concentration induces terminal differentiation of keratinocytes and finally cell death in vitro [21–23]. We examined the effect of a high calcium concentration on keratinocytes in the presence and absence of APC. When exposed to 3.0 mM Ca\(^{2+}\), cultured keratinocytes exhibited morphological changes characteristic of differentiating keratinocytes, including flattening and enlargement of cells and finally cell detachment and cell death. Approximately 50% of keratinocytes survived following 3.0 mM Ca\(^{2+}\) treatment for 24 h (Fig. 5A). In the presence of 20 μg/ml APC, this survival...
rate increased to approximately 70% (Fig. 5A). These data suggest that APC prolongs the survival of terminally differentiated keratinocytes.

Morphological analysis of haematoxylin-stained cells revealed apoptotic changes in cultures treated with 3 mM Ca²⁺. Apoptotic cells were rarely observed (3.5% of total cells) in either control or APC-treated cultures (Figs. 5B-i, iii), whereas loss of adherence and cell apoptosis (indicated by cell shrinkage, small dense basophilic nuclei and reduced nuclear/cyttoplasmic ratio) were rife (more than 30% of total cells) in calcium-treated cultures (Figs. 5B-ii, C). Addition of APC to calcium-treated keratinocytes significantly reduced the number of apoptotic cells (Figs. 5B-iv, C) (\(P < 0.01\)).

When incubated with 3 mM Ca²⁺ for 4 h, keratinocytes expressed much more active caspase-3, a pivotal intracellular protease in apoptotic pathways (Fig. 5D-i). This enzyme was rarely observed in normal cells (data not shown). APC treatment markedly reduced this expression of active caspase-3 (Fig. 5D-ii).

**APC suppresses calcium- and LPS-induced NF-κB activity**

NF-κB is a key regulator of cell death and inflammatory cascades. High activity of NF-κB has been found to inhibit endothelial cell migration [24] and induce cell apoptosis [25]. In this study, untreated or APC-treated human keratinocytes showed low NF-κB activity as detected by EMSA (Fig. 6). Both LPS (100 ng/ml) and calcium (3 mM) strongly activated NF-κB, as indicated by the substantial band shift using EMSA (Fig. 6). APC treatment significantly inhibited LPS and calcium-induced NF-κB activation. A
negative control with labeled DNA probe only and a competitive control with excess unlabelled NF-kB DNA confirmed the specificity of the NF-kB band shift (data not shown).

Discussion

Skin consists of two major tissue layers: a keratinised stratified epidermis and a thick underlying layer of collagen-

Fig. 4. Effect of GM6001 on keratinocyte migration and wound closure stimulated by APC. Wounded keratinocyte monolayers were treated with APC (5 µg/ml), GM6001 (1, 5, 25 µg/ml – G1, G5 and G25) or APC (5 µg/ml) plus GM6001 (APC + G1, APC + G5 and APC + G25). (A) Migration, expressed as % cell migration compared to the control from three independent experiments and (B) wound closure, expressed mean % wound closure ± SEM from three independent experiments, were measured after 24 h. *P < 0.05 and **P < 0.01, compared to control cells at the same timepoint. *P < 0.05 and ++P < 0.01, compared to APC-treated cells.

Fig. 5. Effect of APC on apoptosis. (A) Keratinocyte survival. Keratinocytes were incubated for 24 h under basal conditions (Control) or treated with APC (20 µg/ml), Ca^{2+} (3 mM), or APC plus Ca^{2+}. Cell survival was assessed by trypan blue exclusion and expressed as the mean ± SEM number of live cells per well from three independent experiments. *P < 0.05 compared to control cells and *P < 0.05 compared to Ca^{2+}-treated cells. (B) Haematoxylin-stained photomicrographs showing keratinocytes treated with (i) basal medium, (ii) 3 mM Ca^{2+}, (iii) 20 µg/ml APC, or (iv) Ca^{2+} plus APC for 24 h. Examples of apoptotic cells are arrowed, and exhibit shrinkage, small dense basophilic nuclei and reduced nuclear/cytoplasmic ratios. Scale bar: 25 µM. (C) Results were semi-quantitated by counting the number of apoptotic cells as a percentage of total cells, as described in Methods. Data are expressed as mean ± SEM of three independent experiments. **P < 0.01 compared to control cells and ++P < 0.01 compared to Ca^{2+}-treated cells. (D) Immunostaining of cultured keratinocytes with antibody to active caspase-3. Cells were incubated for 4 h in growth medium containing (i) 3 mM Ca^{2+} or (ii) Ca^{2+} plus APC (20 µg/ml). Scale bar: 25 µM.
rich dermal connective tissue providing support and nourishment. When cutaneous injury occurs, the sheared epidermal edges migrate forward to cover the denuded wound surface and heal the wound [26]. After the initiation of migration, the keratinocytes immediately behind the migrating front undergo a proliferative burst [27,28], which provides a pool of extra cells to replace those lost during injury. Although the function of epidermal keratinocytes is clearly crucial for a wound to heal, the precise signals that stimulate relatively sedentary cell lineages at the wound margin to proliferate, become invasive and then lay down new matrix in the wound gap are unclear. The present study demonstrates for the first time that APC is capable of accelerating in vitro wound closure, possibly by stimulating the proliferation and migration of keratinocytes and prolonging cell survival by preventing keratinocytes from undergoing apoptosis. These data suggest that APC has the potential to promote wound healing by acting on keratinocytes.

APC increased keratinocyte proliferation in a dose-dependent manner between 1 and 20 μg/ml, whilst cell migration responded with a bell-shaped curve, being stimulated at low concentrations (1 and 5 μg/ml) and inhibited at 20 μg/ml. These data suggest that APC-induced proliferation and migration are controlled by distinct cellular mechanisms. Using an in vitro wound healing assay, which involves both cell proliferation and migration, we found that keratinocyte wound closure also exhibited a bell-shape curve in response to different doses of APC, suggesting that wound closure is largely attributable to the migration of keratinocytes. The biphasic migratory response suggests that APC, when used at high concentrations, induces a self-limiting control of cellular signaling, possibly by decreasing and/or recycling APC receptors, as described previously for other growth factors [29,30]. In endothelial cells, the major receptor for APC is endothelial protein C receptor (EPCR) [31] and previous studies have suggested that EPCR-dependent responses to APC are mediated by cleavage of plasminogen-activator receptor (PAR)-1 [32]. We have not directly addressed the receptor and signaling mechanisms involved in APC-induced proliferation and migration, however, these effects are likely to be mediated through the action of APC on related cytokines and MMPs, in addition to its anti-inflammatory function [6,7,32,33].

During wound healing, to cut a path through the fibrin clot or along the interface between clot and healthy dermis, keratinocytes must release their tethers to the basal lamina and dissolve the matrix barrier in their path. Various members of the MMP family, each of which cleaves a specific subset of matrix proteins, are thought to be responsible for this proteolytic activity [34]. Temporal and spatial regulation of MMPs is critical for rapid normal wound healing [35,36], and a disturbed balance of MMPs is often associated with chronically impaired wound healing [37]. The gelatinase sub-group of MMPs, comprising MMP-2 and MMP-9, is extensively involved in several aspects of the wound healing process, including the breakdown of matrix components to allow keratinocyte migration [17,34,38–41]. In this study, we found that APC dramatically elevated the expression and activation of MMP-2, though had no effect on MMP-9. MMP-2 not only regulates the turnover of ECM, but also exerts anti-inflammatory effects via truncation of monocyte chemo-attractant protein (MCP)-3 [42]. MMP-2 is the most efficient MMP in the cleavage and inactivation of MCP-3, which results not only in blocking the initiation of an in vivo inflammatory response but also completely abrogating pre-existing inflammation [42]. Thus, induction by APC of MMP-2 in keratinocytes is likely to promote wound healing not only by promoting cell migration but also by preventing inflammation.

Cytokines are extensively involved in wound healing, regulating inflammation, cell proliferation and migration. In this study, we demonstrate that APC upregulates the pro-
duction of IL-6 and IL-8, which have been reported previously to stimulate keratinocyte proliferation [19,20]. These data are in concert with previous studies showing that APC enhances IL-6 and IL-8 expression in endothelial cells [43] and downregulates the expression of TNF-α in endothelial cells [44], a cytokine which inhibits human keratinocyte proliferation [45]. In addition, upregulation of MMP-2 synthesis and activity by APC may contribute to its effects on keratinocyte proliferation and migration, as MMP-2 is required for keratinocyte migration [17] and stimulates rapid replication of some cell types [46].

Increased proliferation and reduced apoptosis in keratinocytes leads to an accelerated re-epithelialisation of full thickness excisional wounds [47]. Calcium is well established as an extracellular and intracellular modulator of cell proliferation and differentiation in the mammalian epidermis [48,49]. Whereas keratinocytes are best suited to low calcium levels (<0.5 mM) to retain proliferative activity, higher concentrations of calcium suppress proliferation in keratinocytes and lead to the expression of cytokeratins and other markers of differentiation, and finally to cell death [50]. In agreement with previous studies, our data also showed that a high concentration of calcium induces terminal differentiation and apoptosis of keratinocytes. An interesting finding from this study was that APC prolonged the survival of terminally differentiated keratinocytes. This protective function of APC is likely to operate through the prevention of calcium-induced cell apoptosis. APC treatment notably reduced keratinocyte expression of active caspase-3, one of a group of intracellular proteases that are responsible for the systematic disassembly of the cell into apoptotic bodies during apoptosis [51]. Previous studies have shown that APC protects endothelial cells against staurosporine-induced apoptosis [52].

NF-κB is widely recognised as a key regulator of immune and inflammatory responses and has emerged as a decisive factor in the cell’s response to apoptotic challenge [25]. APC’s role in the attenuation of apoptosis in this study is likely to be mediated by inactivation of the NF-κB pathway. The NF-κB pathway’s promotion of apoptosis operates through the activation of death-causing genes, or via indirect upregulation of the expression of other transcription factors that in turn activate genes regulating cell death and/or proliferation. p53 is one such gene and APC has also been shown to protect endothelial cells against p53-mediated apoptosis in ischaemic human brain endothelium [8]. In addition, high activity of NF-κB induced by glucose has been found to inhibit endothelial cell migration [24], suggesting that the regulation of cell migration by APC may be partly through the suppression of NF-κB pathway.

In conclusion, we have demonstrated that APC regulates keratinocyte function in vitro by stimulating proliferation, migration and wound closure and by preventing apoptosis. These events are likely to be due at least partly to APC’s ability to upregulate MMP-2, IL-6 and IL-8 and inhibit NF-κB activity. Taken together, these results demonstrate a central role for APC in modulating keratinocyte function towards a phenotype necessary to promote wound healing.

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