PROTEIN C IS AN AUTOCRINE GROWTH FACTOR FOR HUMAN SKIN KERATINOCYTES

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Running title: Protein C - an autocrine growth factor for keratinocytes

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The protein C (PC) pathway plays an important role in coagulation and inflammation. Many components of the PC pathway have been identified in epidermal keratinocytes including endothelial protein C receptor (EPCR), the specific receptor for PC /activated PC (APC), but the core member of this pathway, PC and its function in keratinocytes has not been defined. In this study, we reveal that PC is strongly expressed by human keratinocytes at both gene and protein levels. When endogenous PC was blocked by siRNA the proliferation of keratinocytes was significantly decreased. This inhibitory effect was restored by the addition of recombinant APC. PC siRNA treatment also increased cell apoptosis by 3-fold and inhibited cell migration by more than 20%. When keratinocytes were pretreated with RCR252, an EPCR blocking antibody, or PD153035, an epidermal growth factor receptor (EGFR) inhibitor, cell proliferation was hindered by more than 30%. These inhibitors also completely abolished recombinant APC (10 μg/ml)-stimulated proliferation. Blocking PC expression or inhibiting its binding to EPCR/EGFR decreased the phosphorylation of ERK2 but increased p38 activation. Furthermore, inhibition of ERK decreased cell proliferation by approximately 30% and completely abolished the APC’s stimulatory effect on proliferation. Taken together, these results indicate that keratinocyte-derived PC promotes cell survival, growth and migration in an autocrine manner via EPCR, EGFR and activation of ERK1/2. Our results highlight a novel role for the PC pathway in normal skin physiology and wound healing.

Protein C (PC), a vitamin K–dependent zymogen, is converted to activated protein C (APC) on the endothelial surface when thrombin binds to thrombomodulin (1;2). The activation of PC is augmented by its specific receptor, endothelial protein C receptor (EPCR) (3-5), a 46-kDa type I transmembrane glycoprotein homologous to major histocompatibility complex class I/CD1 family proteins (6;7). APC plays a key role in the regulation of blood coagulation and also has significant anti-inflammatory properties associated with inhibition of pro-inflammatory cytokines and a reduction of leukocyte recruitment (8;9). APC prevents lipopolysaccharide (LPS)-induced pulmonary vascular injury and protects against ischaemia/reperfusion-induced renal injury by inhibiting the accumulation and activation of leukocytes (10;11). In vitro, APC suppresses the nuclear factor (NF)-κB pathway in both human monocytes (9;12) and endothelial cells (13). APC also inhibits LPS-induced
TNF-α expression in a monocytic cell line (12) and inhibits endothelial cell apoptosis (14). The effectiveness of APC as an anticoagulant and anti-inflammatory agent is demonstrated by its efficacy as a treatment for patients with severe sepsis (15).

APC can activate endothelial matrix metalloproteinase (MMP)-2 (16), a member of the MMP family of zinc-dependent endopeptidases that plays a vital role in the tissue repair process by remodelling the extracellular matrix (17). In cultured human keratinocytes, APC enhanced cell proliferation, migration and MMP-2 activity (18). Recently, a novel function of APC as a promoter of cutaneous wound healing was identified. In a rat healing model APC accelerates full thickness wound closure by stimulating re-epithelialisation, promoting angiogenesis and preventing inflammation (19).

The epidermis is the outermost skin layer and provides the first line of defense against the external environment. Keratinocytes, the predominant cell type in human skin, exist at various stages of differentiation corresponding to different epidermal layers (20;21). Dividing cells in the basal layer progressively differentiate and withdraw from the cell cycle as they are displaced towards 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 producing various mediators including cytokines/chemokines, the T cell receptor and anti-microbial peptides (22). Keratinocytes in the basal and suprabasal layers of the epidermis express many components of the PC pathway, including thrombomodulin (23), PC inhibitor (24) and EPCR (25). Prior to this study, PC was thought to be synthesized almost exclusively by the liver and vascular endothelial cells with a circulatory half-life of ≈20 min (26). The current study shows that PC is strongly expressed by skin keratinocytes. Furthermore, this keratinocyte-derived PC promotes cell survival, growth and migration in an autocrine manner via EPCR, EGFR and activation of ERK1/2.

EXPERIMENTAL PROCEDURES

Keratinocyte culture and reagents - Normal keratinocytes were isolated from neonatal foreskins (n=30) as described previously (18) in accordance with the local ethics regulations. Extracted cells were cultured in keratinocyte-serum free medium (K-SFM, Gibco, Invitrogen Corp., Lakewood, NJ). When greater than 70% confluent, primary cultured cells were trypsinized and used in experiments. Cells were seeded into either 24-well culture plates at 5×10⁵ cells per well or 8-well permanox™ slides (Nalge Nunc International Corp., Rochester, NY) and incubated for 12 hours to allow for adhesion. The confluent cells were then treated with recombinant APC (Xigris, Eli Lilly, Indianapolis, Indiana), PC (Sigma, St Louis, MO), EPCR blocking antibody RCR252, EPCR non-blocking antibody RCR92 (gift from Professor Fukudome, Department of Immunology, Saga Medical School, Nabeshima, Saga, Japan), PD153035, an inhibitor of epidermal growth factor receptor (EGFR), inhibitors of c-Jun (2 μM), p38 (70 nM) and ERK (10 μM) (EMD Biosciences, Inc., San Diego, CA). Medium used for cells treated with PD153035 and related control contained 0.01% dimethylsulphoxide (DMSO) as PD153035 was originally dissolved in
DMSO. MAP kinase inhibitors and PD153035 did not exert any cytotoxic effect on keratinocytes when used at indicated concentrations (data not shown). Cells and culture supernatants were collected for detection of mRNA and protein expression.

Small interfering (si) RNA preparation and nucleofection - Small interfering RNA duplex oligonucleotides were purchased from Proligo (Sigma-Proligo). The designed siRNAs for PC were: sense 5’GAGGUGAGCUUCCUAAUUGC; antisense 5’AAUUGAGGAACCUCCUCUGC. A scrambled form of PC siRNA was used as a negative control. Keratinocytes were adjusted to 1×10⁶ cells/ml in growth medium and subjected to nucleofection using the human keratinocyte nucleofector™ kit and Amaxa nucleofector™ II machine according to the manufacturer’s instructions (Amaxa Biosystems, Cologne, Germany). Cells were allowed to attach overnight and then trypsinised and seeded into either 24 well plates (4 ×10⁵ cells/ml) or 96 well plates (1×10⁴ cells/well) and incubated for 48 and 72 hours. The specificity of siRNAs was confirmed by a validated short hairpin RNA (shRNA) (Superarray, Frederick, MD).

RNA extraction and reverse-transcription (RT)-real time PCR - Total RNA was extracted from keratinocytes using TRI Reagent (Sigma) according to the manufacturer’s instructions. Single stranded cDNA was synthesized from total RNA using AMV reverse transcriptase and Oligo (dT)$_{15}$ as a primer (Promega Corp., Madison, WI). The levels of mRNA were semi-quantified using real time PCR on a Rotor-gene 3000A (Corbett Research, Sydney, Australia). Samples were normalized to the housekeeping gene RPL13A and results were reported for each sample relative to the control. PC PCR product was also separated on a 2% agarose gel and imaged using the Infinity-Capt Gel Documentation System (Vilber, Lourmat, France). Primers used were as follows: PC (213bp): sense 5’TCTTCGTCACCCCAACTAC and anti-sense 5’GGTTTCTTGGCCTCCTTC; RPL13A (152bp): sense 5’AAGCCTACAAAGAAAGTTTGCTATC and anti-sense 5’TGTTCCTGTCAGCCTGTAC.

APC activity assay - The activity of APC in culture supernatants and cell lysates was quantitated using the chromogenic substrate Spectrozyme Pa (American Diagnostica Inc., Stamford, CT) according to the manufacturer's instructions. A standard curve was generated using human recombinant APC.

MTT assay - The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to quantitate the effect of different test agents on cell growth and viability. Briefly, 1×10⁴ cells/well were seeded into a 96-well microplate to a final volume of 200 μl, then incubated for 4 hours to allow cell to attach. Cells were then treated with different test agents. Three hours prior to the completion of the treatment, 10 μl of 5 mg/ml MTT (Sigma) was added to cells. After a further incubation 3 hours, the MTT solution was removed and replaced by 100 μl DMSO. The optical density of each well was determined at a wavelength of 570 nm with a reference wavelength of 630 nm.

In vitro migration assay - Cells were seeded into 24-well plates and cultured to confluence. Cell monolayers were then scratched with a 1000 μl blue plastic pipette tip (Greiner Bio-one, Greiner
International, Longwood, FL), creating a cell-free area approximately 2 mm in width. ‘Wounded’ monolayers were washed twice with PBS to remove loose cell debris, and a defined area of the wound was photographed under phase-contrast microscopy. To standardize the position of wound when photographing small indents were made in the plastic well using sterile 31G needle. To prevent cell proliferation, cells were pretreated with mitomycin C (10 μg/ml, Sigma), which was applied to the cells 2 hours before wounding and removed with three PBS washes. Cell migration was determined after 24 hours by counting the cells that had moved into the wounded area, and the percentage of cell migration was calculated as [number of migrated PC siRNA treated cells / number of migrated scrambled siRNA treated cells] × 100.

**Western blot** - Keratinocytes were washed three times with PBS and lysis buffer (0.15 M NaCl, 0.01 mM PMSF, 1% NP-40, 0.02 M Tris, 6 M urea/H2O) was added. Cell lysates were centrifuged at 10,000g for 15 minutes and supernatants were separated by 10% sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) and transferred to a PDVF membrane. The primary antibodies used were rabbit anti-human EPCR antibody (1:500 dilution, Invitrogen Australia Pty Limited, VIC Australia); mouse anti-human PC (HC-2, 1:500 dilution, Sigma); rabbit anti-phosphorylated forms of p38, c-Jun, and ERK2 (1:1000 dilutions, Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-phospho EGFR (Y1173) and rabbit anti-human active caspase-3 (R&D systems, Minneapolis, MN). Immunoreactivity was detected using the ECL detection system (Amersham, Piscataway, NJ). Anti-human β-actin antibody was included to normalize against unequal loading.

**Immunohistochemical staining** - Cultured keratinocytes in permanox slides were fixed with 4% paraformaldehyde. Human foreskin was fixed with 10% PBS buffered formalin. Paraffin-embedded tissue was de-paraffinised and subjected to immunohistochemistry. After quenching with 2% H2O2 in methanol and equilibrating in PBS, samples were incubated with mouse anti-human PC antibody or mouse IgG (DAKO Corporation, Carpinteria, CA) overnight at 4ºC. Samples were then processed for staining using DAKO LSAB+Systems stain kit (DAKO Corporation) and counterstained with Haematoxylin and Scotts Blueing solution. After mounting, tissue sections were observed under a light microscope (Nikon ECLIPSE 80i, Nikon Corporation, Japan).

For dual-staining, de-paraffinised foreskin tissues were blocked by 5% horse serum in PBS and incubated with goat anti-human EPCR and rabbit anti-human EGFR antibodies (R&D systems) for 2 days at 4ºC. After washing with PBS, tissue sections were incubated with anti-rabbit IgG conjugated with Cy3 (red) and anti-goat IgG conjugated with FITC (green) (1:400, Sigma-Aldrich). Tissue sections were washed with PBS and observed under a fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corporation). Images were acquired and processed using a Nikon digital camera and software (Diagnostic Instruments, Australia) and Image J (http://rsb.info.nih.gov/ij).

**Apoptosis detection** - Apoptotic keratinocytes were detected using an in situ cell death detection kit according to manufacturer’s instructions (Roche Diagnostics Australia Pty. Ltd., NSW, Australia).
Statistical analysis - Significance was determined using one-way ANOVA and Student-Newman-Keuls test. P values less than 0.05 were considered statistically significant.

RESULTS

PC is expressed by human keratinocytes

To investigate whether epidermal keratinocytes produce PC, mRNA from unstimulated primary cultured cells was subjected to RT-PCR. PC mRNA was detected in cultured keratinocytes as shown by the prominent band at 213bp (Fig. 1A). In concordance with gene expression, immunohistochemistry results showed that cultured keratinocyte monolayers stained strongly positive with an antibody against APC/PC (Fig. 1B). The expression of PC was also assessed in neonatal foreskin tissues by immunohistochemistry. The basal and suprabasal layers of the epidermis were strongly positive for PC, whereas the outer stratum corneum where the keratinocytes lose their viability showed weak staining (Fig. 1C). PC was also immunolocalised to dermal vascular endothelial cells (Fig. 1C).

Inhibition of endogenous PC with siRNA suppresses keratinocyte proliferation and migration and promotes apoptosis

Since recombinant APC stimulates keratinocyte proliferation (18), we investigated whether endogenous PC/APC could stimulate the growth of keratinocytes in an autocrine manner. PC siRNA was used to suppress the endogenous PC expression by keratinocytes. The efficacy of PC siRNA was examined at 48 hours by real time PCR and western blot (Fig. 2A&B). PC siRNA dose-dependently reduced PC mRNA levels by up to 80% when used at 0.5 µM. This concentration of siRNA was used in subsequent experiments, unless otherwise specified.

After 24 hours incubation, APC activity (1.53 µg of APC activity/10^6 cells) was detected in the whole cell lysates, but not the culture medium (data not shown), of unstimulated keratinocytes, as measured using the Spectrozyme PCa activity assay. Since the basal culture medium contained no exogenous APC, the activity detected in cell lysates was most likely derived from the activation of endogenous PC. This was confirmed by the finding that the level of APC was reduced by approximately 50% in PC siRNA-treated cells (Fig. 2C).

PC siRNA reduced keratinocyte proliferation by approximately 35% after 72 hours compared to control (Fig. 2D). This inhibition was dose-dependently reversed by adding recombinant APC, with the growth rate being restored at a concentration of 20 µg/ml APC (Fig. 2D).

The effect of PC siRNA on keratinocyte apoptosis was detected, by measuring the amount of active caspase-3, a marker for apoptosis, using western blotting. An 8-fold increase in active caspase-3 was observed in PC siRNA treated cells when compared to that in cells treated with scrambled control (Fig. 3A&B). To confirm this result, apoptotic cells were evaluated by an in situ cell death detection kit. PC siRNA treatment resulted in approximately 3 times more apoptotic cells when compared to scrambled siRNA treatment at 48 hours (Fig. 3C&D).

The migration of PC siRNA treated cells was also measured using a scratch wounding assay. Confluent keratinocytes were scratch-wounded and cell migration was evaluated after wounding for 24 hours. PC siRNA treated cells exhibited ~20% less migration into the wounded areas than control cells (Fig. 4).
EPCR and EGFR are required for endogenous PC-stimulated keratinocyte proliferation

In order to test whether the effect of endogenous PC is mediated through EPCR, unstimulated keratinocytes were treated with RCR252, an antibody which binds to EPCR and prevents PC/APC from binding. After 72 hours, this treatment resulted in a dose-dependent decrease in the proliferation of keratinocytes, showing greater than 30% reduction when cells were treated with 10 μg/ml EPCR (Fig. 5A). These results indicate that keratinocyte-derived PC acts via EPCR to stimulate cell proliferation under normal unstimulated conditions. In addition, the stimulatory effect of recombinant APC on cell proliferation was abrogated by blocking EPCR (Fig. 5A). The non-blocking control antibody, RCR92, had no effect (Fig 5A).

EGFR is highly expressed in human keratinocytes in vivo and in vitro (27;28) and plays a central role in numerous aspects of keratinocyte biology. Functional activation of EGFR results from phosphorylation of specific tyrosine residues in the C-terminal cytoplasmic domain. This activation can be specifically blocked by tyrosine kinase inhibitors such as PD153035. In this study, PD153035 (2.5 μm) inhibited the proliferation of unstimulated keratinocytes by more than 30% (Fig. 5B) suggesting that EGFR is required for normal cell growth. The stimulatory effect of 1 or 10 μg/ml recombinant APC on proliferation was nearly abolished by 2.5 μm PD153035 (Fig. 5B), indicating that APC requires EGFR to stimulate keratinocyte growth. Inhibition of both EPCR and EGFR together caused a further modest, but significant (p<0.05, under basal conditions and in response to 10 μg/ml APC) reduction in proliferation compared to individually blocking either EPCR or EGFR (Fig. 5A&B).

APC upregulates the expression/activation of EPCR and EGFR

When cells were stimulated with 1 or 10 μg/ml recombinant APC, the expression of EPCR and the phosphorylation of EGFR were markedly increased (Fig. 6A&B). In contrast, when cells were treated with PC siRNA, EPCR protein in cell lysates was reduced by more than 50% (Fig. 6A) and the phosphorylated form of EGFR was also dramatically inhibited (Fig. 6B). Thus, APC appears to enhance its effect by increasing the expression/activation of EPCR and EGFR. Using dual immunofluorescent staining, we found that both EPCR and activated EGFR were co-localized in the same areas of basal and suprabasal keratinocytes in the epidermis (Fig. 6C), which is similar to PC localization in skin epidermis (Fig. 1C). Endothelial cells in the dermis stained positively for EPCR, but not for activated EGFR.

APC regulates the activation of MAP kinases

APC-induced cell proliferation is controlled through the activation of MAP kinases in various cell types (18;29). Here, PC siRNA treated keratinocytes were evaluated for their levels of MAP kinase activity. Seventy-two hours after PC siRNA treatment, phosphorylation of ERK2 and c-Jun was attenuated (Fig. 7A&B). In contrast, the phosphorylated form of p38 was increased by approximately 50% (Fig. 7A&B).

Blocking either EPCR with RCR252 (10 μg/ml) or EGFR with PD153035 (2.5 μM) for 24 hours resulted in a similar MAP kinase response as PC siRNA, with a decrease in phosphorylated forms of ERK2,
minimal change in c-Jun and an increase in the phosphorylation of p38 (Fig. 7A&B). Specific inhibitors of ERK, p38 or c-Jun (10 μM, 2 μM, 70 nM respectively) were added to normal cultured keratinocytes for 72 hours in the presence or absence of recombinant APC (1 μg/ml) and cell growth was assessed. Inhibition of ERK decreased cell proliferation by approximately 30% and completely abolished the APC’s stimulatory effect on proliferation. Inhibition of p38 or c-Jun had no effect on cell proliferation (Fig. 7C).

DISCUSSION

Although PC is thought to be synthesized almost exclusively by the liver, the current study shows that it is strongly expressed by skin keratinocytes both in vitro and in vivo. The immunohistochemical localization for PC in the basal and suprabasal layers of the epidermis is similar to the pattern of expression of other members of the PC pathway, including EPCR (25) and thrombomodulin (20). Both these receptors augment the conversion of PC to APC (30;31) in the presence of thrombin, which is also produced by epidermal keratinocytes (32). The ability of keratinocytes to secrete and activate PC is demonstrated by our finding that in the absence of exogenous APC, the Spectrozyme activity assay detected substantial levels of APC in cultured cells which was reduced by treating the cells with PC siRNA (Fig.2C). Thus, the epidermis possesses its own independent PC system which can synthesize PC, activate PC to APC and mediate the function of PC/APC by its receptors/inhibitors.

Keratinocyte growth is thought to be controlled by the autocrine induction of heparin binding factors of the EGF family, by upregulation and dimerization of receptors, and by cross-induction between receptors and ligands (33). Here we show that PC also accounts for an essential part of autocrine growth capacity of cultured human keratinocytes by promoting cell proliferation. In addition to its effect on proliferation, cells treated with PC siRNA had more than a 3-fold higher apoptotic rate than control cells, indicating that PC/APC inhibits apoptosis. PC regulates the activation of caspase-3, a major effector caspase, of which the inactive form is expressed in a wide range of tissues, including the epidermis (34). In normal oral epithelium, cleaved caspase-3 clearly distinguishes apoptotic keratinocytes from cells that are terminally differentiated (35). Recent findings indicate that caspase-14, not caspase-3, is activated during normal keratinocyte differentiation (36). Thus caspase-3 activation appears to be restricted to keratinocytes undergoing apoptosis. Blocking PC by siRNA greatly increased the activation of caspase-3, which is consistent with the increased apoptotic cells.

The PC pathway is a critical regulator of the blood coagulation system and plays an important role in inflammatory and immunomodulatory processes (9;37). Clinical trial results have shown that recombinant APC reduces mortality in patients with sepsis-induced multi-organ failure when administered by infusion (38). Similar to these systemic effects, local production of APC may regulate cutaneous inflammation and immune defence in the skin. Keratinocytes of the epidermis provide the major cellular component of the outermost barrier to the environment (22). When the skin is broken,
a critical response is triggered to restore its protective function. Within 24 hours of wounding, keratinocytes from the wound margins begin to migrate and invade the wound bed, where they proliferate to form the new epithelium. Strong evidence is now emerging that the PC pathway contributes to cutaneous wound repair. In a rat healing model, APC induces angiogenesis and re-epithelialisation whilst inhibiting inflammation to promote cutaneous wound healing (39). In human skin keratinocytes, recombinant APC stimulates proliferation, MMP-2 activity, migration and prevents apoptosis, all vital processes of re-epithelialisation (25). The current study extends on these findings to show that endogenously-derived PC can stimulate a wound healing phenotype in keratinocytes by stimulating proliferation, migration and preventing apoptosis. These results highlight a new important autocrine action of PC/APC during wound re-epithelialisation.

Recent reports show that recombinant APC acts via EPCR to stimulate keratinocyte proliferation (25) and that APC acts via EPCR and EGFR in human peripheral blood lymphocytes to inhibit cell migration (40). In the current study, the decrease in cell growth brought about by inhibitors of EPCR and EGFR could not be recovered by exogenous APC, indicating that endogenous PC/APC promotes cell growth via these two receptors. Simultaneously using inhibitors to both EPCR and EGFR resulted in a modest additional inhibition of proliferation compared to individually blocking either receptor (Fig. 5), suggesting that the two receptors can partially act independently of each other to mediate APC’s proliferative effect. Both EPCR and EGFR are strongly expressed in human skin epidermis (25;41). Dual immunofluorescent staining clearly showed that EPCR and activated EGFR are exclusively co-localized in basal and suprabasal keratinocytes (Fig. 5), similar to the location of PC. Together, these three components comprise a potentially powerful autocrine regulator of the biological functions of keratinocytes.

EGFR, a member of the ErbB family of receptor tyrosine kinases, plays an important role in regulating the development of the epidermis and its appendages (42). In normal epidermis, EGFR is essential for numerous aspects of keratinocyte biology including growth, suppression of terminal differentiation and regulation of cell migration (43). Functional activation of EGFR results from increased phosphorylation of specific tyrosine residues in its C-terminal cytoplasmic domain. In wounded skin, EGFR is transiently up-regulated and is the major effector of the proliferative and migratory responses during wound re-epithelialisation. Accumulating evidence shows that EGFR not only mediates responses to EGF-like ligands, but is also a major transducer of diverse signaling systems and a switch point for cellular communication networks (44;45). PC/APC is not known to act as a ligand for EGFR but it may activate this receptor via other mechanisms, for example by utilizing G-protein coupled receptors (GPCRs). In human keratinocytes, recombinant APC can cleave the GPCR, PAR-1, to stimulate cell proliferation and MMP-2 activity (25). Signalling pathways linking GPCR and EGFR have recently been revealed in some cell types. Sabri et al (46) reported that thrombin activation of PAR-1 in cardiac fibroblasts leads to intracellular transactivation of EGFR, through rapid phosphorylation of phospholipase C, formation of inositol polyphosphate 3 and
mobilization of intracellular calcium resulting in activation of Src and Fyn kinases, which associate with and activate EGRF tyrosine kinase. An alternative pathway termed ‘triple-membrane-passing signal’ (TMPS) has been proposed in which a GPCR activates an ADAM (a disintegrin and metalloprotease), which in turn, releases an EGFR ligand, such as EGF, to activate EGFR (47). Whether endogenous PC/APC acts via these pathways to promote keratinocyte proliferation is yet to be determined.

The MAPK pathway is a prerequisite for growth factor stimulated mitogenesis in many cell types (48). Three major downstream MAPK cascades are mitogen-activated ERK1/2 and stress/cytokine activated p38 and c-Jun N-terminal kinases. Mice lacking Jun in epidermal keratinocytes are born with open eyes (EOB) and eyelid cells and show reduced expression of the EGFR (49). Although c-Jun is primarily a positive regulator of cell proliferation (50), our data show that PC/APC caused minimal change in c-Jun activation in keratinocytes. Instead, 3 lines of evidence indicate that ERK1/2 is the major MAP kinase associated with PC/APC’s enhancement of cell proliferation. Firstly, using siRNA to block keratinocyte-derived PC reduced the activation of ERK. Secondly, preventing PC/APC from binding EPCR or activating EGFR inhibited ERK activation and thirdly, a specific ERK inhibitor prevented PC/APC from inducing proliferation. In contrast, p38 exhibited opposite effects to ERK. These results are in keeping with previous studies which present evidence that p38 MAP kinase functions to promote differentiation and apoptosis whilst signaling through ERK promotes keratinocyte proliferation and survival (51) and endothelial cell proliferation (29).

In summary, the key findings of this study are: i) PC is strongly expressed by keratinocytes, the major cell type of the skin epidermis, ii) Endogenous PC/APC stimulates proliferation and migration and inhibits apoptosis in keratinocytes in an autocrine manner and iii) Stimulation of proliferation by endogenous PC/APC is mediated via two receptors, EPCR and EGFR and the MAP kinase ERK1/2. These novel findings highlight the importance of the PC pathway in skin physiology and help elucidate keratinocyte function in normal wound healing.

REFERENCES


10


**FOOTNOTES**

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The abbreviations used are: APC, activated protein C; EPCR, endothelial protein C receptor; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; MMP, matrix metalloproteinase; EGFR, epidermal growth factor receptor; MTT, colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; siRAN, small interfering RNA.

FIGURE LEGENDS

Fig. 1. Expression of human PC/APC in keratinocytes and neonatal foreskin.
A) PC mRNA expression by primary human keratinocytes was detected by RT-PCR. Left lane, DNA markers. B) PC protein was detected in human keratinocytes by immunohistochemistry using mouse anti-human PC antibody. Scale bar: 40 μm. Mouse IgG was used as a negative control. C) PC protein was detected in human neonatal foreskin. Black arrows indicate vascular endothelial cells. Arrow heads indicate epidermal basal layer. Images represent one of three independent experiments. Scale bar: 100 μm.

Fig. 2. PC siRNA treatment inhibits the growth of keratinocytes
A) Efficacy of PC siRNA at blocking PC mRNA expression by keratinocytes at 48 hours, detected by RT-real time PCR. Data are expressed as mean ± SEM (n=3). B) PC/APC levels in the supernatant and cell lysate of keratinocytes following PC siRNA treatment for 48 hours detected by western blot. C) APC activity in cell lysates after treatment for 48 hours with either scrambled control siRNA or PC siRNA (0.5 µM). D) Growth rate of keratinocytes in response to PC siRNA (0.5 µM) and APC (0.1, 1, 10 and 20 µg) treatment after 72 hours as detected by MTT assay. Cell proliferation is expressed as a percentage of control (mean ± SD). Graphs represent one of three independent experiments. * P<0.05, ** P<0.01.

Fig. 3. Endogenous PC/APC prevents keratinocyte apoptosis.
Keratinocytes were treated with PC siRNA (0.5 µM). After 48 hours, cells were harvested and cell lysates were used to detect the activation of caspase-3 by A) western blot and B) semi-quantified using image analysis software. C) Apoptotic cells (black arrows indicate apoptotic cells) were detected using in situ cell death detection kit and D) quantitated by counting apoptotic cells under high power microscopy (×20). Data were expressed as the average number of apoptotic cells per field out of 15 fields (mean ± SEM, n=3). Images represent one of three independent experiments. * P<0.05. ** P<0.01. Scale bar: 40 μm.

Fig. 4. PC siRNA treatment decreases migration of keratinocytes.
Cell monolayers were pretreated with mitomycin C and then scratched with a 1000-μl blue plastic pipette tip. The defined area of the wound was photographed under a phase contrast microscopy at time 0 (0h). To standardize the position of the wound for photography, small indents were made in the plastic well (marked as block arrows). Cell migration was determined after 24 hours by counting cells that had moved out of the initial area, and the percentage of cell migration calculated as [number of migrated PC siRNA treated cells / number of migrated scrambled siRNA treated cells] × 100. Data are expressed as the
number of migrated cells as a percentage of control (mean ± SEM, n=3). Images represent one of three independent experiments. *\( P<0.05 \). Scale bar: 100 µm.

**Fig. 5.** Blocking EPCR and EGFR decreases the growth of unstimulated and APC-stimulated keratinocytes.

Cell proliferation was detected using MTT assay. A) The growth rate of keratinocytes in response to the EPCR blocking antibody, RCR252, or control non-blocking antibody, RCR92, in the presence or absence of APC (1 µg/ml) after 72 hours. B) Cells were incubated in keratinocyte-serum free medium without EGF and treated with PD153035, an inhibitor to EGFR or PD153035 plus RCR252, 1 hour prior to addition of recombinant APC (1 or 10 µg). After incubation for 72 hours, cell proliferation was measured by MTT assay. Data are expressed as a percentage of control cell proliferation (mean ± SD) and graphs represent one of three independent experiments. * indicates comparison with normal control. + indicates comparison with APC treatment. ♣ indicates blocking both EPCR and EGFR in comparison with individual treatment. ♣, + or *\( P<0.05 \), ++ or **\( P<0.01 \).

**Fig. 6.** EPCR and EGFR are co-localised in skin and upregulated by PC/APC in keratinocytes.

Keratinocytes were treated with recombinant APC or PC siRNA and the expression of A) EPCR and B) the phosphorylated form of EGFR (P-EGFR) were detected in whole cell lysates by western blot. C) Co-localization of activated EGFR and EPCR on foreskin epidermis. EGFR: Red, EPCR: Green. Merged image: Yellow indicates co-localisation of EPCR and EGFR. White arrows indicate basal epidermis and arrow heads indicate vascular endothelium. Images represent one of three independent experiments. Scale bar: 50 µm

**Fig. 7.** PC/APC regulates the activation of MAP kinases.

A) Keratinocytes were treated with PC siRNA (0.5 µM) for 72 hours or RCR252 (10 µg/ml), PD153035 (2.5 µM) for 24 hours and cells collected for western blot analysis. Images represent one of three independent experiments. B) Western blotting results were analysed by image analysis software. Data are expressed as a percentage of control protein expression (Mean± SEM, n=3). *\( P<0.05 \), ** \( P<0.01 \). C) Keratinocyte proliferation was measured at 72 hours in response to inhibitors of c-Jun (2 µM), p38 (70 nM) and ERK (10 µM). Data are expressed as a percentage of control cell proliferation (mean ± SD). Images represent one of three independent experiments. *\( P<0.05 \), ** \( P<0.01 \).
FIGURES

Figure 1

A

B

C

D

200bp→ PC

PC

IgG

PC

IgG

PC

IgG
Figure 2

A

PC mRNA (% control)

PC siRNA (µM)

0          0.1         0.2         0.5

B

PC siRNA (µM)

PC mRNA (% control)

0          0.5         0.5        0.5         0.5         0.5    APC (µg/ml)

0             0          0.1          1          10           20

C

APC activity (% control)

Control   PC siRNA

D

Cell proliferation (% control)

0          0.5          0.5          0.5          1          10          20

PC siRNA (µM)

APC (µg/ml)

†††
Figure 4

A

Control PC siRNA (0.5 µM)

0h

24h

B

Migrated cells (% control)

Control PC siRNA

*
Figure 5

A

Cell proliferation (% control)

0 0.1 1 10 0 0.1 1 1 10 0 RCR252 (μg/ml)

0 0 0 0 1 1 1 1 1 10 APC (μg/ml)

0 0 0 0 0 0 0 0 0 10 RCR92 (μg/ml)

B

Cell proliferation (% control)

0 2.5 2.5 0 2.5 2.5 0 2.5 2.5 2.5 PD153035 (μM)

0 0 10 0 0 10 0 0 10 10 RCR252 (μg/ml)

0 0 0 1 1 1 10 10 10 APC (μg/ml)
Figure 6

A

<table>
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<th>APC (µg/ml)</th>
<th>PC siRNA (µM)</th>
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EPCR

β-actin

B

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<td>0.5</td>
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P-EGFR

β-actin

C

EPCR

P-EGFR

Merged Image
Protein C is an autocrine growth factor for human skin keratinocytes
Meilang Xue, David Campbell and Christopher J. Jackson

J. Biol. Chem. published online February 9, 2007

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