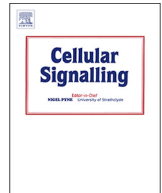
Cellular Signalling 47 (2018) 37–43

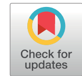
Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



Thrombin promotes PAI-1 expression and migration in keratinocytes via ERK dependent Smad linker region phosphorylation



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A B S T R A C T

Keratinocyte proliferation and migration is essential during re-epithelialisation for the restoration of the epithelial barrier during skin wound healing. Numerous growth factors are involved in the stimulation of keratinocyte proliferation and migration. The signalling pathways that drive these processes during wound healing are not well defined. This study investigated thrombin-mediated signalling in keratinocytes. The thrombin receptor, protease-activated receptor 1 (PAR-1) is a seven transmembrane G-protein coupled receptor that is known to transactivate the epidermal growth factor receptor (EGFR). Immortalized human keratinocytes (HaCaT cells) were treated with thrombin and selective inhibitors to EGFR and MAP kinases. Whole cell lysates were separated on SDS-PAGE and analysed by Western blot using antibodies against transcription factor Smad2. Quantitative real-time polymerase chain reaction was used to measure the mRNA expression of PAI-1 while scratch wound assays were used to measure keratinocyte migration. Western blot data showed that thrombin mediates PAR-1 transactivation of EGFR and the downstream phosphorylation of the transcription factor Smad2 linker (Smad2L) region. ERK1/2 inhibition by UO126 caused a decrease in Smad2L phosphorylation while the p38 inhibitor SB202190 and JNK inhibitor SP600125 did not. Smad2L Ser250 was specifically phosphorylated by this thrombin mediated pathway while Ser245 and Ser255 were not. Thrombin increased PAI-1 mRNA expression and keratinocyte migration and this was reduced when either EGFR or ERK1/2 were blocked. Taken together these results show that thrombin mediated mRNA expression of PAI-1 in keratinocytes and migration occurs via EGFR transactivation and involves signalling intermediates ERK1/2 and Smad2 and may be a key pathway in skin wound healing.

1. Introduction

In the early coagulation phase of skin wound healing the serine protease thrombin plays a key role in clot formation to mediate the release of leukocyte chemoattractants and activators [1]. Wound repair then progresses through the inflammatory and growth phases with epidermal keratinocyte proliferation and migration across the wound bed enabling re-epithelialisation and restoration of skin barrier function [2,3]. Thrombin also acts as a signalling molecule in keratinocytes activating its G-protein coupled receptor PAR-1 (proteinase activated receptor 1) [4]. PAR-1 can transactivate the epidermal growth factor receptor (EGFR) by metalloproteinase (MMP)-mediated release of heparin-binding EGF-like growth factor (HB-EGF) [5,6]. Activated EGFR promotes keratinocyte proliferation and migration via Fyn-mediated tyrosine phosphorylation of integrin β4 resulting in disruption of hemidesmosomes and the detachment of keratinocytes for subsequent migration [2,7]. In oral keratinocytes, EGF increases extracellular signal-regulated kinase (ERK1/2) phosphorylation of the linker region of transcription factor Smad2 to regulate the duration of TGFβ signalling [8]. The serpin plasminogen activator inhibitor type 1 (PAI-1) is an extracellular matrix protease inhibitor that facilitates extracellular matrix remodeling and cell migration during wound healing [9,10]. Following an injury PAI-1 is induced in keratinocytes and aids efficient cell migration [11]. To date thrombin-mediated PAI-1 expression has been reported in vascular endothelial cells [12] and mesangial cells [13]. Here we have investigated thrombin-mediated PAI-1 signalling in keratinocytes as a potential mechanism contributing to wound healing. Our data shows that in keratinocytes thrombin mediates PAR-1 transactivation of EGFR and this results in activation of ERK1/2 and subsequent phosphorylation of the linker region of Smad2 specifically on

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https://doi.org/10.1016/j.cellsig.2018.03.009
Received 28 August 2017; Received in revised form 19 February 2018; Accepted 20 March 2018
Available online 22 March 2018
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Ser250. This signalling pathway drives both PAI-1 gene expression and keratinocyte migration.

2. Materials

Materials used in this study were as follows: RPMI media, trypsin–EDTA, penicillin and streptomycin (Gibco, MA, USA), foetal bovine serum (FBS) (In Vitro Technologies, VIC, Australia), human epidermal growth factor (EGF) and thrombin (Sigma-Aldrich, MO, USA), human transforming growth factor–β (TGFβ) (Cell Signalling Technology, MA, USA), 30% acrylamide/bis, ammonium persulphate, Tween 20, Trans-Blot Turbo RTA Transfer Kit and PVDF membrane (Bio-Rad, CA, USA), TEMED, TRIS and glycine (Amresco, TX, USA), MagicMark™ XP (Invitrogen, MA, USA). UO126 (MEK inhibitor, Promega, Australia), SB202190 (p38MAPK inhibitor), SP600125 (JNK inhibitor), AG1478 (EGFR inhibitor) (Sigma-Aldrich). Primary antibodies anti- phosphoSmad2 (Ser245) rabbit IgG polyclonal, anti-phosphoSmad2 (Ser250) rabbit IgG polyclonal and anti-phosphoSmad2 (Ser255) rabbit IgG polyclonal were kindly gifted by Professor Koichi Matsuzaki (Kansai Medical University, Japan), anti-phosphoSmad2(Ser465/467) and anti-phosphoSmad2 (Ser245/250/255), secondary antibody anti-rabbit IgG horseradish peroxidase (HRP) conjugate and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP conjugate antibody (Cell Signalling Technology). Chemiluminescence ECL detection kit (GE Healthcare UK), bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, VIC, Australia), RNeasy Mini kit, QuantiTect Reverse Transcription kit, quantifast SYBR Green, Hs SERPINE1 (PAI-1) and 18 s ribosomal RNA primers (Qiagen, Melbourne, Australia).

3. Methods

3.1. Cell culture

Flasks of confluent HaCaT cells grown in RPMI containing 10% (v/v) heat-inactivated FBS and 1% penicillin and streptomycin in CO_2 (5%) incubator at 37 °C. For experiments cells were trypsinized, diluted with media and centrifuged at 1000 *g* for 5 min at room temperature. The cell pellet was resuspended and cells counted on a Beckman Z series Coulter counter. Cells were seeded in complete media at 5×10^5 cells/60 mm dish and once confluent they were serum-deprived (RPMI containing 0.1% (v/v) heat-inactivated FBS and 1% penicillin and streptomycin) for 24 h before experimentation. For some experiments inhibitors UO126, SB202190, SP600125, AG1478 or 0.05% DMSO were added to the culture media 30 min prior to addition of agonist either thrombin (0–30 U/ml, 1 h), EGF (100 nM, 15 min), or TGFβ (2 ng/ml) for the times indicated.

3.2. Cell scratch model

HaCaT cells were grown to confluence in 24-well plates and serum-deprived for 24 h. A line gap, mimicking an incisional wound in vivo, was made at the centre of each well by scratching using a sterile 200 μl pipette tip. Cells were then washed with PBS before fresh complete medium and treatments were added as indicated in the text. Pictures of cells were taken by phase-contrast microscopy (Nikon VIC, Australia) every hour after the scratch for 24 h and NIH Image J image software was used for recording and measurement of cell migration across the scratch gap and digital image analysis.

3.3. Quantitative real-time RT-PCR (qRT-PCR)

PAI-1 mRNA expression was measured using qRT-PCR. In each well of 12 well plate 10^5 cells were plated and after 6 h or time as indicated total RNA was extracted using RNeasy kit. The purity and concentration of the isolated RNA was determined using a NanoDrop 2000 spectrophotometer. First strand cDNA was synthesised using QuantiTect Reverse Transcription Kit. qRT-PCR was performed using Quantifast SYBR Green PCR kit on a Rotor-Gene Q. Hs PAI-1primers were used and data were normalised to 18 s housekeeping gene. Relative expression of PAI-1 mRNA was calculated by the ΔΔCt method.

3.4. Western blot analysis

Whole cell lysates (30 μg protein) were separated on 10% SDS-PAGE and transferred to PVDF membranes. For protein band detection membranes were blocked with 5% (w/v) BSA in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween-20) at room temperature for 1 h and then were incubated with primary antibodies as indicated (1:1000 dilution in 5% (w/v) BSA in TBST, unless otherwise stated) overnight in 4 °C with gentle rocking. Membranes were briefly washed then incubated with secondary antibody HRP conjugate (1:4000 in 5% (w/v) BSA in TBST) for 1 h at room temperature. Protein bands were visualised by ECL on a ChemiDoc XRS+ and digital images were processed using ImageLab™ software (Bio-Rad).

3.5. Statistical analysis

All experiments were repeated at least three times. One-way ANOVA followed by post-tests as required were performed using GraphPad Prism (GraphPad Software). Data is shown as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical significance was set at $p < 0.05$.

4. Results

4.1. Thrombin mediates Smad2 linker region phosphorylation via EGFR transactivation pathway in keratinocytes

Previously we have reported thrombin signalling capable of mediating phosphorylation of Smad transcription factors in vascular smooth muscle cells [14]. Here we examined whether thrombin could similarly trigger phosphorylation of the Smad2 linker (Smad2L) region in keratinocytes. Western blotting (Fig. 1) showed thrombin (2 U/ml) stimulated a 1.8-fold increase in pSmad2L (Ser245/250/255) phosphorylation ($p < 0.01$) while EGF treatment (100 nM) resulted in a 2.8-fold increase ($p < 0.01$) relative to untreated controls. Levels of total Smad2 did not change under any test treatments. In order to determine whether the thrombin-mediated phosphorylation occurred via transactivation of EGFR we pre-treated the cells with AG1478 (5 μM). This concentration is known to effectively inhibit EGFR mediated Smad signalling [15]. AG1478 completely blocked thrombin and EGF-mediated pSmad2L (Ser245/250/255) phosphorylation ($p < .01$) (Fig. 1). Thrombin time course experiments (Fig. 2) showed pSmad2L (Ser245/250/255) phosphorylation levels increased 3–4 fold within 30 min and remained elevated for at least 4 h. These results demonstrate that in keratinocytes thrombin can elicit PAR1-EGFR transactivation that leads to sustained Smad2 linker region phosphorylation.

4.2. Thrombin mediated phosphorylation of Smad2L by MAPKs in keratinocytes

As EGF treatment is known to increase ERK1/2 phosphorylation of Smad2L in keratinocytes [8] we investigated if thrombin can trigger MAPK phosphorylation of Smad2L. Cells were stimulated with thrombin in the presence or absence of the following inhibitors: UO126 (3 μM) inhibits MEK1/2 which prevents ERK1/2 activity, SB202190 (3 μM) inhibits p38 MAPK, SP600125 (1 μM) inhibits JNK activation (Fig. 3). Thrombin increased pSmad2L (Ser245/250/255) levels ($p < .01$) by 2.7-fold which was completely blocked by UO126 ($p < .01$). SB202190 and SP600125 had no effect on thrombin stimulated Smad2L (Ser245/250/255) phosphorylation. These results

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