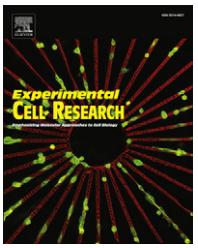
EXPERIMENTAL CELL RESEARCH 319 (2013) 390–401

Available online at www.sciencedirect.com

SciVerse ScienceDirect

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



Research Article

Cell motility in models of wounded human skin is improved by Gap27 despite raised glucose, insulin and IGFBP-5

Catherine S. Wright[a], *Rebecca F. Berends*[a], *David J. Flint*[b], *Patricia E.M. Martin*[a,*]

[a]*Department of Life Sciences, School of Health and Life Sciences, Glasgow Caledonian University, 70 Cowcaddens Road, Glasgow G4 0BA, UK*
[b]*Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK*

ARTICLE INFORMATION

Article Chronology:
Received 30 May 2012
Received in revised form
12 December 2012
Accepted 13 December 2012
Available online 20 December 2012

Keywords:
Connexin
Peptide
IGF
Keratinocyte
Skin
Wound

ABSTRACT

Reducing Cx43 expression stimulates skin wound healing. This is mimicked in models when Cx43 function is blocked by the connexin mimetic peptide Gap27. IGF-I also stimulates wound healing with IGFBP-5 attenuating its actions. Further, the IGF-I to IGFBP-5 ratio is altered in diabetic skin, where wound closure is impaired. We investigated whether Gap27 remains effective in augmenting scrape-wound closure in human skin wound models simulating diabetes-induced changes, using culture conditions with raised glucose, insulin and IGFBP-5. Gap27 increased scrape-wound closure in normal glucose and insulin (NGI) and to a lesser extent in high glucose and insulin (HGI). IGF-I enhanced scrape-wound closure in keratinocytes whereas IGFBP-5 inhibited this response. Gap27 overcame the inhibitory effects of IGFBP-5 on IGF-I activity. Connexin-mediated communication (CMC) was reduced in HGI, despite raised Cx43, and Gap27 significantly decreased CMC in NGI and HGI. IGF-I and IGFBP-5 did not affect CMC. IGF-I increased keratinocyte proliferation in NGI, and Gap27 increased proliferation in NGI to a greater extent than in HGI. We conclude that IGF-I and Gap27 stimulate scrape-wound closure by independent mechanisms with Gap27 inhibiting Cx43 function. Gap27 can enhance wound closure in diabetic conditions, irrespective of the IGF-I:IGFBP-5 balance.

Introduction

Following acute skin wounding, the gap junction protein connexin43 (Cx43) is reduced at wound margins. By contrast, in non-healing diabetic wounds Cx43 is upregulated at wound edges and is associated with delayed wound closure [1,2]. Gap junctions are channels that link the cytoplasm of adjoining cells, composed of two opposing hemichannels, each formed from six transmembrane connexin proteins. Up to ten connexin subtypes are expressed in skin [3]. Gap junctions allow direct exchange of small molecules (<1 kDa) such as cAMP between cells. Connexins can also form non-junctional hemichannels [4] which have adhesive properties involved in cell spreading and migration [5,6].

Agents that reduce Cx43 gene expression accelerate wound closure in both normal mice [7,8] and diabetic rats [9]. This correlates with our recent *in vitro* finding that connexin mimetic peptides (CMPs), which functionally block connexin-mediated communication, rather than affecting connexin gene expression, increase scrape-wound closure in human epidermal keratinocytes, dermal fibroblasts and organotypic skin models [10,11].

Abbreviations: CMC, Connexin-mediated communication; CMP, Connexin mimetic peptide; Cx43, Connexin43; ECM, Extracellular matrix; GJIC, Gap junctional intercellular communication; HGI, High glucose and insulin; IGFBP-5, Insulin-like growth factor binding protein 5; NGI, Normal glucose and insulin; ODN, Oligodeoxyribonucleotide

*Corresponding author. Fax: +44 141 331 3208.
E-mail address: Patricia.Martin@gcu.ac.uk (P.E.M. Martin).

0014-4827/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yexcr.2012.12.013

CMPs, expressly designed to inhibit connexin signalling [12], mirror short sequences in the two extracellular loops of connexins, and are reversible, effective experimental tools [13]. The CMP Gap27 has been shown to be Cx43-selective and effective in increasing human keratinocyte and dermal fibroblast migration [10]. However, the mechanisms by which connexins influence wound closure, following reduction of connexin-mediated communication (CMC) via gap junctions or hemichannels, remain to be elucidated.

IGF-I, a mitogenic survival factor for many cells in the body, can increase cell migration and thus influence wound healing [14]. In human skin IGF-I is expressed by dermal fibroblasts and keratinocytes of the stratum granulosum, with keratinocytes in the basal layer expressing the IGF-I receptor [15]. IGF-I is upregulated in normally-healing wounds [16], but downregulated in diabetic wounds [17]. Wound healing is accelerated in mice over-expressing IGF-I [18], and transplantation of keratinocytes over-expressing IGF-I increases wound closure in a diabetic porcine model [19]. IGF-I actions are modulated by a family of IGF binding proteins, IGFBP-1-6 (see [20] for review). Of these, IGFBP-5 is increased in diabetes [21,22], and IGFBP-5-mediated cell migration is inhibited by hyperglycaemia [23]. However, the role of IGFBP-5 in these models is unclear as IGFBP-5 has been proposed to both inhibit and augment IGF-I actions. Furthermore, recent studies have linked IGFBP-5 to fibrosis, including scleroderma. IGFBP-5 activates lung and dermal fibroblasts, as well as liver stellate cells, inducing fibrotic responses similar to that seen with TGF-β1 [24,25]. Thus IGFBP-5 could be an important inhibitor of wound healing by limiting bioactive IGF-I and stimulating scar formation.

This study advances our previous work by investigating whether the CMP Gap27 remains effective in augmenting scrape-wound closure in 3D and 2D human skin models cultured in conditions mimicking type II diabetes where glucose and insulin are raised, and IGF-I:IGFBP-5 ratios are altered. The outcomes will allow us to predict the efficacy of Gap27 in a more complex environment, such as non-healing diabetic wounds, where hyperglycaemia, hyperinsulinaemia and disturbance of growth factors contribute to chronic wound creation.

Materials and methods

Cell sources, culture and additives

Primary human dermal fibroblasts were derived as previously described [10] from discarded paediatric foreskins following informed consent with ethical approval by Yorkhill Hospital Trust Research Ethics Committee, Glasgow. Fibroblast monolayers were maintained in euglycaemic DMEM containing 5.5 mM glucose supplemented with 10% v/v FBS, 2 mM glutamine, 50 U/mL penicillin and 50 U/mL streptomycin (Lonza, Wokingham, UK). Primary human keratinocytes were purchased from Invitrogen (Paisley, UK) and maintained in Epilife® medium containing 5.5 mM glucose with 1% v/v Epilife defined growth supplement (formulation proprietary), 10 μg/mL gentamicin and 0.25 μg/mL amphotericin B (Invitrogen). Recombinant mouse IGFBP-5, which has a 97% conservation with human IGFBP-5, was expressed and purified as described previously [26], and recombinant human IGF-I was obtained from R&D Systems Europe Ltd (Abingdon, UK). The CMP ^{43}Gap27 used in this study mimics amino acids 204–214 (SRPTEKTIFII) on extracellular loop 2 of Cx43 [12] (Zealand Pharma, Glostrup, Denmark). Scrambled Gap27 (TFEPDRISITK; Severn Biotech, Kidderminster, UK) [27] was used as a control, and CMPs were reconstituted as previously described [10].

Glucose and insulin pre-conditioning of cells and viability assay

To mimic 'normal' and 'type II diabetic' levels of glucose and insulin in the skin [28,29], following plating, cells were treated daily for a minimum of 5 days with 5.5 mM glucose and 1 nM insulin (normal glucose and insulin (NGI) conditions), or 25 mM glucose and 10 nM insulin (high glucose and insulin (HGI) conditions) in DMEM or Epilife® prior to experiments being carried out. This allowed pre-conditioning of the cells to these environments. To ensure cell viability was maintained following NGI and HGI pre-conditioning, cells (1×10^5/ml) were plated onto cover-chambers and treated as above. Cells were stained with 2.5 μM calcein-AM and 1 μM propidium iodide (both Invitrogen) to respectively determine viable and dead cells numbers, and observed on a Zeiss confocal microscope attached to a LSM510 laser scanning system. Images were acquired and processed as previously described [10]. Cells were >90% viable regardless of treatment. Three dimensional human organotypic cultures were formed as described previously [11]. Organotypic cultures were also pre-conditioned for 5 days in NGI or HGI conditions prior to experiments being performed.

Keratinocyte and organotypic scrape-wound cell migration assays

Keratinocytes were seeded at 1×10^5/well in 12-well plates, and grown to confluent monolayers in NGI or HGI. Cells were treated with 100 μM Gap27 or scrambled Gap27 (control) in Epilife® containing NGI or HGI in the presence or absence of 100 ng/ml IGF-I and/or 4 μg/ml IGFBP-5. After treatment, scrape-wounds were introduced and cell migration monitored as previously described [10]. Scrape-wounds of around 600 μm were introduced to confluent cell monolayers with a 100 μL pipette tip, and cell migration into the wound monitored by taking triplicate measurements of the scrape-wound width at 0, 6, 24, 30 and 48 hours. Cell migration was monitored using a CMEX-3200 camera and measurements were made with ImageFocus© software (Euromex, Anhem, Netherlands). Peptide-containing media was replenished every 12 h, and experiments were performed three times ($n=3$) in triplicate per treatment group. Organotypic scrape-wound assays were carried out in a similar fashion, with the scrape being carefully introduced into the epidermal layer [11] ($n=3$).

Western blotting

Cells (1×10^6) were plated onto six-well plates, grown to sub-confluence, and pre-conditioned with NGI or HGI for 5 days. Cells were then harvested and Western blotting carried out as described by Easton et al., 2009 [30]. Primary antibodies were a rabbit polyclonal Cx43 antibody directed against the carboxyl tail (diluted 1:5000, a kind gift from Dr Edgar Rivedal, University of