Biomaterials 31 (2010) 2542–2548

Contents lists available at ScienceDirect

Biomaterials

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

# The effects of TGF-α, IL-1β and PDGF on fibroblast adhesion to ECM-derived matrix and KGF gene expression

Xintong Wang [a,1], Heather Waldeck [b,2], Weiyuan J. Kao [a,b,c,*]

[a] School of Pharmacy, University of Wisconsin-Madison, 777 Highland Ave, Madison, WI 53705, USA
[b] Department of Biomedical Engineering, College of Engineering, University of Wisconsin-Madison, WI, USA
[c] Department of Surgery, School of Medicine and Public Health, University of Wisconsin-Madison, WI, USA

ARTICLE INFO

*Article history:*
Received 10 September 2009
Accepted 7 December 2009
Available online 29 December 2009

*Keywords:*
Keratinocyte growth factor
Granulocyte-macrophage colony-stimulating factor
Interpenetrating network
Interleukin-1β
Transforming growth factor-α
Platelet-derived growth factor-BB

ABSTRACT

The goal of this study was to elucidate the control mechanisms by which exogenous proteins regulate keratinocyte growth factor (KGF) expression in fibroblasts adhered to differing substrates and thereby provide insights into both fundamental *in vitro* cell signaling and cell-biomaterial interaction research. A serum-free culture system in which cells maintained their proliferative capacity was established and employed. The addition of transforming growth factor- α (TGF-α), interleukin-1β (IL-1β) and platelet-derived growth factor-BB (PDGF-BB) individually showed no effect on KGF protein release, however, IL-1β addition led to increased KGF mRNA transcription, intracellular KGF protein synthesis, and granulocyte-macrophage colony-stimulating factor (GM-CSF) release. Intracellular KGF protein synthesis and extracellular release were enhanced when fibroblasts were treated with a combination of IL-1β and PDGF-BB which suggests KGF synthesis and release are largely regulated by synergistic mechanisms. Surface-bound fibronectin-derived ligands and individual exogenous proteins promoted fibroblast adhesion to semi-interpenetrating polymer networks (sIPNs) but did not stimulate KGF release despite enhancement of KGF mRNA transcription. Additionally, serum conditioning was found to have a significant impact on KGF synthesis and the subsequent mechanisms controlling KGF release. This study demonstrates that KGF release from fibroblasts is likely regulated by multiple mechanisms involving post-transcriptional and exocytic controls which may be impacted by the presence of serum and how serum is removed from the *in vitro* cell environment.



## 1. Introduction

Mammalian fibroblast growth factors (FGFs) form a large family of polypeptide growth factors which function in cell signaling, proliferation and differentiation [1–3]. Keratinocyte growth factor/fibroblast growth factor-7 (KGF/FGF-7) has been shown to be secreted by mesenchymal cells with mitogenic activity on keratinocytes [1]. In a wound healing environment, KGF is produced via fibroblast–keratinocyte paracrine interaction[4–6] and subsequently enhances the proliferation and migration of dermal keratinocytes [7,8]. KGF also influences epithelial cell differentiation and regulates the expression of wound remodeling proteases [9,10]. A wide range of growth factors and cytokines able to induce KGF expression, such as transforming growth factor-α (TGF-α), interleukin-1β (IL-1β), platelet-derived growth factor-BB (PDGF-BB) and tumor necrosis factor-α (TNF-α), have been reported [1,11–13]. Most of these studies, however, are based on conditions using serum-containing media and a tissue culture polystyrene (TCPS) substrate, thereby reflecting the combined consequences of the various serum components and the likely substrate-specific effects of a single type of surface. Moreover, the majority of published reports on the effects of growth factors on KGF expression involve cells transferred from serum-containing media directly into serum-free media without any type of gradual transition period [11–13]. Although viability of the cells used in these studies is unclear, such culture conditions result in a sudden change of environment which may lead to stress-induced functional differences. Furthermore, previous work has not drawn a connection between KGF gene transcription and subsequent protein synthesis and/or release. While serial transitioning of cells from serum-containing into serum-free media has been shown to be selectively accompanied by up- or down-regulation of certain genes, demonstrating a fine-tuning of signaling pathways, [14,15]

* Corresponding author. School of Pharmacy, University of Wisconsin-Madison, 777 Highland Ave, Madison, WI 53705, USA. Tel.: +1 608 263 2998; fax: +1 608 262 5345.
*E-mail addresses:* xwang47@wisc.edu (X. Wang), hmwaldeck@wisc.edu (H. Waldeck), wjkao@pharmacy.wisc.edu (W.J. Kao).
[1] Tel.: +1 608 262 4680; fax: +1 608 262 5345.
[2] Tel.: +1 608 262 4616; fax: +1 608 262 5345.

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.12.018

there is an overall deficiency in studies comparing the cell behavior in abruptly versus serially transitioned serum-free cultures.

In this study, human dermal fibroblasts successfully adapted into serum-free media were seeded onto differing surfaces with or without modification with adhesive peptide motifs. Fibronectin-derived peptides arginine-glycine-aspartic acid (RGD) and proline-histidine-serine-arginine-asparagine (PHSRN) were employed as they have shown synergistic effects on cell adhesion, migration, proliferation and protein expression, and have previouly been utilized to modify biomaterials [16–18]. These peptides were conjugated to a semi-interpenetrating polymer network (sIPN) based on poly(ethylene glycol) (PEG) and gelatin to gain mechanistic insights into our previous studies, where we had shown that the sIPN modified with ECM-derived ligands enhanced cell adhesion and influenced cytokine release in fibroblast-keratinocyte and fibroblast-monocyte coculture systems as well as promoted ECM remodeling in rodent full-thickness dermal defect and porcine partial-thickness defect models *in vivo* [6,19–22]. The use of dissimilar substrates also allowed examination of whether similar KGF control mechanisms were present when fibroblasts were adhered to different surfaces. The effects of exogenous proteins on adhesion and protein release were analyzed in cells which were serially transitioned into serum-free media. The resulting variations in KGF transcription and protein release depending on exogenous protein or serum stimulation provide insights into the control mechanisms governing KGF release by fibroblasts and the development of tissue engineering strategies for wound regeneration.

## 2. Materials and methods

### 2.1. Synthesis of sIPNs containing PEGylated fibronectin-derived peptides

RGD and PHSRN were prepared by solid phase peptide synthesis utilizing Fmoc chemistry. Peptide formation was confirmed by $^1$H NMR and matrix assisted laser desorption/ionization (MALDI, University of Wisconsin School of Pharmacy) with 95% purity confirmed with HPLC. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Synthesis and characterization of PEGylated-peptide modified gelatin has been described previously [23]. Briefly, terminal hydroxyl groups of PEG diol (2 kDa, Fluka) were converted to carboxylic acid with the addition of t-butyl bromoacetate and dioxane, and then reacted with *N*-hydroxysuccinimide to synthesize PEG-bis-*N*-hydroxysuccinimide (PEG-bis-NSu). Glycine-glycine-glycine (GGG, Fluka), RGD, and PHSRN were grafted onto one of the two terminal groups of PEG-bis-NSu by the addition of *N*,*N*-diisopropyl ethylamine to form *N*-hydroxysuccinimide-PEG-ligand. Gelatin was grafted to the other terminal group by adding *N*-hydroxysuccinimide-PEG-ligand to 1% gelatin in phosphate buffered saline (PBS) solution (pH = 8) with *N*,*N*-diisopropyl ethylamine. The final product was purified by a pressurized ultrafiltration system (Millipore, Bedford, MA) with a 30 kDa membrane followed by lyophilization. The degree of peptide-PEG grafting onto gelatin was analyzed by trinitrobenzenesulfonic acid-based spectrophotometry [19,20]. The percent modification of gelatin was approximately 70 percent for GGG modified gelatin, 66 percent for RGD modified gelatin and 71 percent for PHSRN modified gelatin.

sIPNs were prepared by combining 575 Da poly(ethylene glycol) diacrylate (PEGdA) and PEGylated-peptide grafted gelatin or unmodified gelatin dissolved in $ddH_2O$ to create a 13 w/w% PEGdA, 9 w/w% gelatin solution. The initiator, 2,2-dimethoxy-2-phenyl-acetophenone, was incorporated into the PEGdA and gelatin solution to a final concentration of 0.1 wt%. The solution was poured into 9.5 mm diameter Teflon® molds and crosslinked with UV light (CF 1000, Clearstone Technologies, Inc., Minneapolis, MN) at 365 nm for 3 min. sIPNs were sterilized with 70% ethanol for 30 min and washed three times with PBS before treatment with antibiotics (Penicillin 300 IU/mL, Streptomycin 300 μg/mL and Amphotericin B 0.75 μg/mL, Mediatech, Inc., Herndon, VA) overnight [24]. sIPNs were then washed three times with PBS for 15 min each rinse before being fitted into 48-well plates and equilibrated in serum-free fibroblast basal media (FBM, Lonza, Walkersville, MD) at 37 °C for 2 h. The FBM was then replaced for cell seeding.

### 2.2. Cell culture and transitioning to serum-free media

Neonatal human dermal fibroblasts (NHDF) were obtained from Lonza and cultured in fibroblast growth medium-2 (FGM-2, Lonza) containing FBM, 0.1% insulin, 0.1% recombinant human fibroblast growth factor-B, 0.1% GA-1000, and 2% fetal bovine serum (FBS). Fibroblasts for all experiments were derived from the same cell stock at passage 6–8 and cells were harvested and split into each experimental group at 80 to 90% confluence.

To successfully study the efficacy of serially transitioning cells to serum-free media, cells were split into two groups. One group contained cells which were passaged directly into serum-free media [FBM or Dulbecco's Minimum Essential Media:Ham's Nutrient Mixture F-12, 1:1 Mix (DMEM/F-12, Mediatech)]. In the other group, over the course of three passages, the serum concentration was slowly reduced by serially adjusting the ratio of FGM-2 to the new media at each passage (from 1:1 to 1:3 to 1:9 and then to serum-free media). DMEM/F12 supplemented with 5% FBS (DMEM/F12 + 5%FBS) was also used as a serum-containing media control. Cells from both groups were seeded onto 48-well tissue culture polystyrene (TCPS, Becton Dickinson Labware, NJ) at a seeding density of 10,000 cells/cm$^2$. Adherent cell density was quantified and cell viability was observed at 24, 72, 120, and 168 h using the method described below.

### 2.3. Cell adhesion and viability studies by LIVE/DEAD viability/cytotoxicity assay

Adherent cell density was quantified by LIVE/DEAD viability/cytotoxicity assay for mammalian cells (Invitrogen, Eugen, OR). Cells were washed twice with Dulbecco's PBS (D-PBS, Mediatech) followed by addition of 2 μM calcein-AM and 2 μM ethidium homodimer-1 and then incubated at 37 °C for 30 min. Cell morphology and viability were then observed using an inverted microscope (Nikon Eclipse TE 300) and images were recorded for cell counting. Five images per sample were taken at random fields of view.

### 2.4. KGF and GM-CSF expression and release studies by ELISA

To study the effect of serum on KGF protein release, cells were cultured on TCPS in FBM, FBM with 2% FBS, FBM with 5% FBS or FGM-2 at a cell seeding density of 50,000 cell/cm$^2$. Cell supernatant was assayed for KGF at 24, 72, 120, and 168 h using an enzyme-linked immunosorbent assay (ELISA, Quantikine KGF immunoassay colorimetric kit, R&D Systems, Minneapolis, MN).

In order to study the effects of individual exogenous proteins on cell adhesion as well as KGF and granulocyte-macrophage colony-stimulating factor (GM-CSF) release, serum-free FBM was supplemented with one of the following human recombinant proteins: TGF-α (BioVision, Mountain View, CA), IL-1β (BioVision), PDGF-BB (Sigma–Aldrich) or a combination of IL-1β and PDGF-BB. A concentration of 10 ng/mL of each growth factor or cytokine was used based on literature showing enhanced fibroblast KGF mRNA expression at this concentration [11–13]. After serial media transition, fibroblasts were seeded onto TCPS and sIPNs at a seeding density of 50,000 cells/cm$^2$. FBM supplemented with 5% FBS served as a positive control. Cell culture supernatant was collected for ELISA (Quantikine KGF and GM-CSF immunoassay colorimetric kits, R&D Systems) and adherent cell densities were quantified with LIVE/DEAD assay at 24, 72, 120 and 168 h.

Cell lysate studies were performed to gain insights into protein release and extracellular transport. Cells were seeded onto 6-well TCPS plates with or without sIPNs at a density of 50,000 cells/cm$^2$ in serum-free FBM or FBM supplemented with 10 ng/mL IL-1β, 10 ng/mL IL-1β and 10 ng/mL PDGF-BB, or 5% FBS. At 48 and 120 h, cells were washed with cold D-PBS and lysed in mammalian protein extract reagent (M-PER, Pierce, Rockford, IL) supplemented with EDTA-free Halt™ protease inhibitor cocktail (Pierce). After centrifugation at 7000 RPM for 5 min, the KGF concentration in the supernatant was analyzed via ELISA. The total protein of cells cultured on TCPS was determined by BCA™ protein assay (Pierce) and the KGF content in cell lysate was presented as a ratio of KGF to total protein concentration. An accurate measure of total protein from cells adhered to sIPNs could not be obtained due to the presence of gelatin in the sIPN.

### 2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Semi-quantification of KGF mRNA was achieved with RT-PCR. All chemicals and reagents used for RT-PCR were obtained from Invitrogen (Carlsbad, CA) unless otherwise stated. After serial media transition, fibroblasts were seeded onto sIPNs and TCPS at a density of 50,000 cells/cm$^2$ in FBM, FBM plus 10 ng/ml IL-1β, or FBM supplemented with 5% FBS. At 24, 72 and 120 h, Cells were washed with D-PBS twice and total RNA was collected using TRIzol® reagent according to the manufacturer's recommendations. The RNA concentration and purity was determined by NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). 1 μg of total RNA from each TCPS sample and 600 ng of total RNA from each sIPN sample were treated with DNase I (amplification grade). cDNA was produced using the Moloney murine leukemia virus (M-MLV) reverse transcriptase system and 200 ng per 1 μg total RNA of 15-mer random primers (University of Wisconsin Biotechnology Center) was used in place of random hexamers. KGF and RNA polymerase II (RPII) primers (University of Wisconsin Biotechnology Center) were designed to bind to human cDNA using Primer3Plus software [25] based on DNA sequences obtained from the NCBI database (Bethesda, MD) (Table 1). RPII has been shown to have stable RNA transcription in various tissues and experimental conditions making it an appropriate choice for a reference gene for this study [26]. PCR was