Regulation of keratinocyte growth factor gene expression in human skin fibroblasts

Aimin Tang, Barbara A. Gilchrest*

Department of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA

Received 12 December 1994; revision received 14 February 1995; accepted 8 March 1995

Abstract

Human keratinocyte growth factor (KGF) is a recently identified mitogen for epithelial cells produced by normal stromal fibroblasts. KGF has been shown to stimulate keratinocyte migration and promote re-epithelialization of skin suggesting a critical role for KGF in wound healing. To understand how KGF might be regulated during wound healing, we examined the ability of the pro-inflammatory cytokines interleukin-1α (IL-1α), interleukin-1β (IL-1β) interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) transforming growth factor-β1 (TGF-β1) and interferon-γ (IFN-γ) to modulate KGF gene expression in cultured human fibroblasts, using northern blot analysis. Exposure to IL-1α (20 units/ml) or IL-1β (100 units/ml) for 24 h increased KGF mRNA expression by 352% and 504%, respectively, with early induction seen at 2 h and maximal induction seen at 8 h. TNF-α (30 ng/ml) increased KGF mRNA expression by 535% at 24 h, with induction first seen at 8 h. The maximal induction of KGF mRNA was observed when IL-1α, IL-1β and TNF-α were used at 100 units/ml, 100 units/ml and 3 ng/ml, respectively, although concentrations 100–500-fold lower (IL-1α, 0.02 units/ml; IL-1β, 0.02 units/ml; and TNF-α, 0.03 ng/ml) were nearly as stimulatory, increasing KGF mRNA expression by 175%, 254% and 322%, respectively. IL-6 (200 units/ml), TGF-β1 (5 ng/ml) and IFN-γ (200 units/ml) did not change the level of KGF mRNA at 24 h in human fibroblasts under the same conditions. The protein synthesis inhibitor cycloheximide abrogated the effects of IL-1α, IL-1β and TNF-α on KGF gene induction, indicating that new protein synthesis is required in the process. Dexamethasone (10⁻⁷ M), known to inhibit inflammatory reactions and retard wound healing, also inhibited the induction of KGF mRNA expression by IL-1α, IL-1β and TNF-α. Individual variation in KGF mRNA expression was seen when fibroblasts from different aged donors were analysed, but no consistent age-associated change was observed. These results suggest that IL-1α, IL-1β and TNF-α up-regulate KGF gene expression in fibroblasts and might be responsible for its induction following skin wounding or other injury.

Keywords: Keratinocyte growth factor; Fibroblasts; IL-1/TNF-α

* Corresponding author, Tel.: (617) 638 5538; Fax: (617) 638 5550.

Abbreviations: IL-1, interleukin-1; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; KGF, keratinocyte growth factor; TNF-α, tumor necrosis factor-α; transforming growth factor-β1, TGF-β1; interferon-γ, IFN-γ.

1. Introduction

Cutaneous wound healing is a complex process consisting of inflammatory reactions, formation
of granulation tissue re-epithelialization and tissue remodeling [1]. A large number of growth factors and cytokines have been identified to be involved in these processes [2–3]. Keratinocyte growth factor (KGF) is a recently identified growth factor which plays an important role in skin wound healing [4–7].

KGF (alternatively designated FGF7) belongs to the fibroblast growth factor (FGF) family which include acidic FGF (FGF1) basic FGF (FGF2), Int-2 (FGF3), HST (FGF4), FGF5 and FGF6 [8–10]. The gene for human KGF consists of 3 coding exons and 2 introns, and is transcribed into a predominant 2.4 kb transcript and a less abundant 5 kb transcript [11]. The product of KGF gene, a monomeric 28 kDa polypeptide, exerts its effects by binding and activating a specific cell surface KGF receptor [12]. KGF is unique among the FGF family in that it is strongly mitogenic for epithelial cells including keratinocytes, but not stimulatory for fibroblasts or endothelial cells [8–9]. KGF message is found exclusively in the dermis of skin, suggesting that it has a paracrine effect on the growth of overlying epithelial tissues [9]. Skin injury induces a significant increase in KGF mRNA expression within 24 h [4]. KGF stimulates migration and proliferation of keratinocytes in vitro [13] and application of purified KGF to skin wounds significantly increases the rate of re-epithelialization and dermal regeneration [5–6]. The blockage of KGF function by expression of a dominant-negative KGF receptor transgene in basal keratinocytes leads to epidermal atrophy in the skin of transgenic mice, reduces the proliferation rate of epidermal keratinocytes at the wound edge and results in substantially delayed re-epithelialization of the wound upon skin injury [7], lending further support for the importance of KGF in wound healing.

Because of the role of KGF in wound healing, it is important to understand how its expression is regulated. In this study, we investigated the roles of pro-inflammatory cytokines including interleukin-1α (IL-1α), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and dexamethasone and aging known to have negative effects on wound healing [14–18], in KGF gene expression by northern blot analysis. We show that IL-1α IL-1β and TNF-α up-regulate KGF gene expression in a time- and dose-dependent fashion, that the modulation of KGF gene expression by these cytokines requires de novo protein synthesis, and that dexamethasone suppresses this cytokine-induced KGF gene expression. We also show that aging, although a deleterious factor in wound healing, has no effect on the constitutive expression of KGF mRNA in fibroblasts.

2. Materials and methods

2.1. Cell culture

Primary dermal fibroblast cultures were established from newborn foreskin or adult skin by the outgrowth method as described previously [19] and maintained in DMEM (Gibco BRL Life Technologies Inc., Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT). Second or third passage of confluent fibroblasts were used for experiments.

2.2. Reagents

Human recombinant IL-1α and human recombinant IL-1β were purchased from Becton Dickinson Labware (Bedford, MA). Recombinant human interleukin-6 (IL-6) and recombinant human TNF-α were purchased from Biosource International (Camarillo CA). Ultra-pure natural human transforming growth factor-β1 (TGF-β1) was obtained from Genzyme (Cambridge, MA). Recombinant human interferon gamma (IFN-γ) was purchased from Endogen (Boston, MA). They were dissolved in culture medium and diluted before use. Cycloheximide was purchased from Sigma (St. Louis, MO).

2.3. RNA extraction

Total RNA was isolated from human fibroblasts using TRI REAGENT (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer’s protocol. RNA concentration was determined by spectrophotometry.
2.4. PCR amplification and cDNA probe preparation

A cDNA probe for human KGF was generated using RT-PCR as described before [20]. In brief, 1 μg of RNA was reverse-transcribed and amplified using a GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT). Sense (5'-GCTCTACAGATCAT-GCTTTCCAC-3') and antisense (5'-CTTCATTGCATTTCTITTGTGC-3') KGF primers encompassed the coding region (nucleotides 365-732) of mRNA for human KGF [9]. Primers were used at 1 μM for both cDNA synthesis and amplification. PCR parameters included denaturation at 95°C for 1 min, annealing at 55°C for 1.15 min and polymerization at 72°C for 2 min for 35 cycles. After the last cycle of amplification, the samples were incubated for 7 min at 72°C. PCR products were separated on a 1.5% low melting point agarose gel (GIBCO BRL) and the expected cDNA band in size of 367 base pairs was extracted using QIAEX gel extraction kit (QIAGEN Chatsworth, CA) according to the manufacturer's protocol. Purified cDNA for KGF was then directly labeled with [32P] dCTP and used as probe. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control cDNA probe was obtained from Clontech Laboratories Inc. (Palo Alto CA). Radiolabeled probes were generated from 50–100 ng cDNA using a [32P] dCTP (New England Nuclear, Boston, MA) and an oligonucleotide-primed DNA labeling kit (Pharmacia) following the instructions of the manufacturer. The unincorporated [32P] dCTP was separated from the labeled DNA by centrifugation through a G-50 mini-spin column as recommended by the manufacturer (Worthington Biochemical Corp. Freehold, NJ). Incorporated radioactivity was assayed using a scintillation counter (Pharmacia).

2.5. Northern blot analysis

Total cellular RNAs were extracted using TRI REAGENT as described above. Fifteen-to-twenty-micrograms of RNAs with O.D. 260/280 ratio greater than 1.8 were size fractionated in 1% agarose/6% formaldehyde gels, and transferred onto Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL) which were hybridized with 1–2 × 10⁶ counts/min per ml of 32P-labeled KGF probe and washed as described [21]. The membranes were then rehybridized with the G3PDH cDNA probe to assess uniformity of RNA loading and transfer. After being air dried, the membranes were autoradiographed with Kodak XAR film and signals were analysed using a densitometer. Densitometric readings are expressed as the ratio of experimental signal to G3PDH.

3. Results

3.1. IL-1α, IL-1β and TNF-α up-regulate KGF gene expression

Skin injury has been shown to stimulate KGF gene expression in vivo and maximal induction was observed within 24 h [4]. To determine whether pro-inflammatory cytokines are involved in the regulation of KGF gene expression after injury, we first examined the effects of various cytokines on KGF mRNA expression in cultured human foreskin fibroblasts. Fibroblasts were incubated with IL-1α, IL-1β, IL-6, TNF-α, TGF-β1 and IFN-γ for 24 h. RNA was prepared at the end of treatment and analysed by northern blotting. IL-1α, IL-1β and TNF-α increased the level of KGF mRNA in fibroblasts to 352%, 504% and 535%, respectively over that of control cells while little change was observed when fibroblasts were treated with IL-6 (122%), TGF-131 (90%) or IFN-γ (140%) (Fig. 1). We focused on IL-1α, IL-1β and TNF-α in the following experiments. The kinetic study showed that the induction of KGF gene expression in fibroblasts was seen as early as 2 h, the first timepoint examined, for IL-1α (176%) and IL-1β (291%). The KGF gene expression was dramatically increased at 4 h and at 8 h, but had returned toward the baseline by 24 h. In contrast, TNF-α induced KGF gene expression more gradually and less dramatically, with the highest level of KGF mRNA in fibroblasts to 352%, 504% and 535%, respectively over that of control cells while little change was observed when fibroblasts were treated with IL-6 (122%), TGF-131 (90%) or IFN-γ (140%) (Fig. 1). We focused on IL-1α, IL-1β and TNF-α in the following experiments. The kinetic study showed that the induction of KGF gene expression in fibroblasts was seen as early as 2 h, the first timepoint examined, for IL-1α (176%) and IL-1β (291%). The KGF gene expression was dramatically increased at 4 h and at 8 h, but had returned toward the baseline by 24 h. In contrast, TNF-α induced KGF gene expression more gradually and less dramatically, with the highest level of KGF mRNA expression observed at 24 h (Fig. 2). A dose-response study revealed that both IL-1α and IL-1β at concentrations as low as 0.02 units/ml caused increases in KGF gene expression to 175% and 254% of the basal level. The significant induction of KGF mRNA was observed at concentrations of 2–100 units/ml for IL-1α and 0.2–100 units/ml for IL-
TNF-α at 0.03 ng/ml increased KGF mRNA expression to 322% of control, with the maximal induction of twice this level seen at a concentration of 3 ng/ml (Fig. 3). No synergetic or additive effects were observed between IL-1α and IL-1β, IL-1α and TNF-α or IL-1β and TNF-α (Fig. 4).

3.2. Protein synthesis is required for KGF induction by cytokines

To determine if protein synthesis is required in the process of KGF mRNA induction by IL-1α, IL-1β and TNF-α, fibroblasts were treated with the protein synthesis inhibitor cycloheximide for 30 min before incubation with the cytokines. KGF mRNA induction by IL-1α, IL-1β and TNF-α was inhibited by 81% 70% and 64%, respectively (Fig. 5), indicating that de novo protein synthesis is involved in the induction of KGF gene expression by these cytokines. Slight inhibition (22%) of constitutive KGF mRNA expression was seen in control cells treated with cycloheximide (Fig. 5).

3.3. Dexamethasone inhibits KGF induction by IL-1α, IL-1β and TNF-α

Corticosteroids are potent inflammatory inhibitors and have been shown to retard wound healing and specifically block the function of IL-1α, IL-1β and TNF-α [14–17,22–25]. To determine whether glucocorticoids could modulate the induction of KGF mRNA by cytokines, dexamethasone (10⁻⁷ M) was added to cultures together with IL-1α, IL-1β or TNF-α. Dexamethasone caused 46, 76 and 80% inhibition of KGF mRNA induction by IL-1α, IL-1β and TNF-α, respectively compared to paired cultures not treated with dexamethasone while its effect on the constitutive level of KGF mRNA was minimal (Fig. 6), suggesting a negative mechanism in the regulation of KGF gene expression.
3.4. Aging has no effect on constitutive KGF gene expression

It is well known that aging has deleterious effects on wound healing [18]. To determine whether aging could affect KGF production, we examined KGF mRNA expression in fibroblasts derived from newborn foreskin and adult skin biopsies. All cultures were studied at post primary passage level 2 or 3 under identical conditions, as noted in 'Materials and methods', and harvested 24–72 h after the last medium change. Substantial individual variation in KGF mRNA expression was observed (Fig. 7), with no suggestion of a feeding effect. There was no consistent age-associated change: both the highest and lowest KGF mRNA levels were observed in adult samples, with newborns being intermediate in expression.

4. Discussion

Cytokines are important mediators of normal wound healing. Following tissue injury a large number of cytokines including IL-1 and TNF-α are released from inflammatory cells platelets, endothelial cells and keratinocytes [26–27]. IL-1 and TNF-α are multifunctional cytokines exerting a wide range of biological effects in immune response, hematopoiesis and inflammation [28]. Significant levels of IL-1 and TNF-α are actually detected in wound fluid in both animal models and patients [29–30], indicating a role of these cytokines in wound healing. In vitro, IL-1 has been shown to stimulate the growth of fibroblasts.
and keratinocytes, collagen production by fibroblasts, and chemotaxis of keratinocytes [27,31–33]. IL-1 has also been reported to stimulate the production of human hepatocyte growth factor (HGF) [34], a broad spectrum growth factor for hepatocytes, endothelial cells and epithelial cells by fibroblasts in vitro. HGF is involved in liver regeneration [34]. In a porcine skin wound model, Sauder et al. observed that IL-1 significantly enhanced the process of healing and normal epidermal regeneration [35]. In this report, we demonstrate that IL-1α and IL-1β induce KGF gene expression in human fibroblasts, which might be responsible for the role of IL-1 in epidermal regeneration of skin wound reported by Sauder et al. [35]. Available data suggest that TNF-α may also have a role in wound healing or remodeling. In vitro TNF-α modulates fibroblast synthetic activities, including inhibition of collagen and fibronectin production and stimulation of collagenase and prostaglandin E₂ secretion [36–38]. Mooney et al. [39] observed an increase in wound-breaking strength after topical TNF-α administration in both normal and wound-impaired animals; and Bettinger et al. [40] also reported that TNF-α might decrease wound tensile strength, using an in vivo mouse model. In this study, we demonstrate that TNF-α increases KGF gene expression in human fibroblasts. Since KGF has been shown to be a strong stimulant for keratinocyte proliferation and migration in vitro [13], and to enhance re-epithelialization in a skin wound model in vivo [5–6], our result suggests that TNF-α, through stimulating KGF expression, also has positive effects on wound healing. TNF-α stimulation of human HGF production may be a second mechanism by which TNF-α stimulates wound healing [34].

Our results concerning IL-1 in the induction of KGF gene expression agree with the data published by Chedid et al. [41] during the preparation of this manuscript. In their paper Chedid et al. [41] showed that IL-1 induces KGF mRNA expression and protein production in a dose- and time-dependent manner, that IL-1-treated fibroblast conditioned medium stimulates keratinocyte proliferation, and that anti-KGF antibody inhibits this proliferation [41]. However, we disagree on the effects of TNF-α and IL-6 on KGF expression. We find that TNF-α increases KGF gene expression, an effect inhibited by dexamethasone and cycloheximide, similar to the KGF induction by IL-1α and IL-1β, and that IL-6 has little effect. Chedid et al. report that IL-6 increases KGF gene expression and keratinocytes, collagen production by fibroblasts, and chemotaxis of keratinocytes [27,31–33].
expression and that TNF-α had no effect [41]. There are several possible explanations for the discrepancy. Firstly, we used human foreskin fibroblasts, while they used human embryonic lung fibroblasts. The postnatal versus embryonic status of the cells, as well as their different sites of origin, might affect cytokine response. Differential responses of human embryonic lung fibroblasts and human gingival fibroblasts to TNF-α have been documented by Tamura et al. in the induction of human HGF [34], for example. Secondly, different culture conditions were used. We used serum containing medium that might have masked a subtle effect of IL-6 on the induction of KGF gene as reported by Chedid et al. who used serum-free medium in their fibroblast cultures. Similarly, IL-6 concentrations higher or lower than the 200 ng/ml we selected might have influenced KGF expression in our experiments.

The exact mechanism of induction of KGF mRNA by IL-1α IL-1β and TNF-α is not clear. Chedid et al. demonstrated by nuclear run on assays that the increase in KGF transcript level following IL-1 stimulation was due to an increased rate of KGF gene transcription [41]. We show that the protein synthesis inhibitor cycloheximide blocks the induction of KGF mRNA by IL-1α, IL-1β and TNF-α, consistent with the involvement of new transcription factor(s) or nuclear factor(s) such as NF-κB, an intracellular messenger believed to transmit the gene induction signal from the cytoplasm to the nucleus and known to be involved in IL-6 gene induction by IL-1 and TNF-α [42–44]. More studies are needed to elucidate fully of the mechanism(s) involved in KGF gene regulation.

Corticosteroids are widely used therapeutic agents that have a major side effect of impairing wound healing [14]. Although the exact mechanism by which corticosteroids suppress wound healing is not known, pharmaceutical levels of corticosteroids have been shown to retard wound healing by impairing the chemotaxis of inflammatory cells (an early part of the repair process), inhibiting angiogenesis, and decreasing fibroblast proliferation and matrix synthesis [17]. Systemic administration of TGF-β1 reverses some aspects of corticosteroid-impaired wound healing in rats [45]. Our study shows that 10⁻⁷ M dexamethasone, a physiological dose [23–24], significantly inhibits the induction of KGF mRNA by IL-1α, IL-1β and TNF-α, indicating that the inhibition of cytokine-induced KGF expression might be at least partially responsible for the adverse effects of corticosteroids on wound healing and that administration of exogenous KGF might be able to improve the process of wound healing in corticosteroid-treated patients.

Aging is a well-recognized factor affecting wound healing. In the elderly, all phases of the
wound healing process are affected [18]. Studies in both human and animal models of wound healing suggest that aging is accompanied by decreased inflammatory and proliferative responses, delayed angiogenesis, delayed remodeling, and slower re-epithelialization [18]. Using a transgenic mouse model, Werner et al. [7] demonstrated that the blocking of KGF function by expression of a dominant-negative KGF receptor transgene in basal keratinocytes causes age-dependent phenotypic abnormalities in the transgenic mouse skin that are characterized by epidermal atrophy, abnormal morphology of hair follicles, and dermal hyperthickening, indicating a possible dysfunction of KGF in the elderly. We did not find an age-associated change in constitutive expression of the KGF gene in our study, suggesting that at least basal KGF production in the elderly might not be decreased. Similarly, Compton et al. [46] recently reported that gene expression of TGF-β1 (a cytokine with significant potential for modulation of connective tissues in wound healing) in cultured human keratinocytes was not affected by aging. We did not examine the possibility that aging might affect KGF induction by cytokines. Also, we cannot exclude the possibility that aging affects the expression and function of cell surface receptors for KGF, as recently demonstrated for epidermal growth factor in our laboratory [47] and suggested by the study of Werner et al. [7]. It is also possible that skin fibroblasts from different anatomic locations have different basal levels of KGF mRNA, in that we examined fibroblasts from newborn foreskin versus adult arm or trunk skin, although in the small number of donors studied mRNA levels in our older adults were in fact higher than in younger adults.

In summary, the present study demonstrates that IL-1α, IL-1β, and TNF-α induce KGF gene expression in human skin fibroblasts in vitro via a process that requires de novo protein synthesis and that dexamethasone exerts a negative effect on this cytokine-induced KGF gene expression. These findings contribute to our understanding of the complex interactions between cytokines and growth factors during tissue homeostasis and wound healing.

Acknowledgements

The authors thank Dr. Wende R. Reenstra for helpful discussions and Kristin Ostrom for excellent technical support.

References


[31] Tamura M, Arakaki N, Tsubouchi H, Takada H, Daikuhara Y: Enhancement of human hepatocyte growth factor production by interleukin-1α and -1β and tumor...


