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Cloning and Expression of Human Keratinocyte Growth Factor in *Escherichia coli* for Recombinant Drug Production

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Background: Keratinocyte growth factor (KGF) is a member of fibroblast growth factor (FGF) family which induces proliferation and differentiation in a wide variety of epithelial tissues. KGF plays an important role in protection, repair of various types of epithelial cells, and re-epithelialization of wounds. Therefore, in patients with hematologic malignancies receiving high doses of chemotherapy and radiotherapy, treatment with KGF decreases the incidence and duration of severe oral mucositis.
Objectives: The aim of this study was to express the recombinant form of human keratinocyte growth factor in *Escherichia coli*.
Materials and Methods: *KGF* gene was amplified by PCR and cloned into the expression vector pET28a(+). The recombinant vectors were transformed into *E. coli* BL21(DE3) as expression host and expression of the desired protein was induced by IPTG. The expression was evaluated at RNA and protein levels by reverse transcriptase PCR (RT-PCR) and SDS-PAGE analyses, respectively and the expressed protein was confirmed through western blotting.
Results: Cloning was confirmed by PCR and restriction digestion. RT-PCR and SDS-PAGE represented expression of KGF in *E. coli*. The optimized expression was achieved 16 hours after induction with 0.3 mM IPTG at 37°C in luria broth (LB) containing kanamycin. The 18 kDa protein was confirmed by western blotting, using anti-His antibodies.
Conclusions: The result of the present study indicated that *E. coli* expression system was suitable for overexpression of recombinant human KGF and the expressed protein can be considered as a homemade product.

*Keywords:*Cloning; Recombinant Protein; Keratinocyte Growth Factor

1. Background

Oral mucositis results from injury of normal oral epithelium, exposed to toxic agents, in patients with hematologic malignancies receiving chemotherapy and radiotherapy. Oral mucositis is one of the common problems in patients with cancer undergoing chemotherapy (1). Keratinocyte growth factor (KGF) is an epithelial cell-specific growth factor and plays a role in proliferation, migration and morphogenesis of these cells. Palifermin as a recombinant form of human KGF, due to its ability to cause proliferation of the oral mucosa and protect against mucosal injury, is a treatment option for high-risk patients for oral mucositis, specifically ones with hematological malignancies receiving stem cell transplantation. Mucositis is also a problem for patients receiving treatment for nonhematological tumors. Palifermin is the first FDA-approved drug which reduces the incidence and duration of severe oral mucositis by protecting those cells and stimulating the growth of new epithelial cells, to build up the mucosal barrier in patients with hematologic malignancies (2). KGF is a member of fibroblast growth factor family. FGFs influence proliferation and differentiation of various cell types.

The *KGF* gene contains three exsons and an intron, located in 15q13-q22 of chromosome 15. The KGF cDNA encodes a 194-amino acid protein. Its 31 N-terminal amino acids are separated to produce the active and secretory form of the protein. The first 23 amino acid residues of KGF could be removed without decreasing biological activity, so when human keratinocyte growth factor expressed in E.coli a protein with molecular size 18 kDa was obtained (3). KGF binds to FGFR2b isoform, its specific receptor expressed predominantly by epithelial cells, and they exert their biological activities as follows:

- Stimulating mitogenic activity in a variety of epithelial cells (4).
- Increased migration of normal keratinocytes after epithelial tissue injury (5).

Implication for health policy/practice/research/medical education:
In order to make a home-made recombinant human KGF, PET system and E. coli could be applied by Iranian researchers. As our knowledge, this is the first project in our country about this manner.

• Role in the early stages of differentiation (6).
• Morphogenesis of epithelium and re-epithelialization of wounds (7).
• Antiapoptotic effects (8).
• Cellular protective effects on epithelial cells (9).

Since KGF affects proliferation and differentiation of epithelial cells, it is used for treatment of pathologies in liver, lungs, gastrointestinal tract, and especially wound healing, in many tissues and organs (2, 9). Due to specificity of KGF and its receptor in epithelial tissues and upregulation of KGF after epithelial injury, there is a focus to identify its clinical applications in cases that the integrity of epithelial surfaces are at risk (4, 10).

2. Objectives

Due to therapeutical importance of recombinant KGF, the aim of this study was to clone and overexpress the recombinant form of human KGF for the first time in Iran.

3. Materials and Methods

3.1. Gene Amplification

The synthetic *KGF* gene sequence including restriction enzyme recognition sites and His-tag signal, was designed. The KGF cDNA was amplified using Taq DNA polymerase and following primers: (F: 5́- gataccatgggccatcatcatcatcatcatc -3́; R: 5́- ccgcctctcgagttaagttattgccataggaa -3́). The synthetic KGF cDNA was used as a template. Amplification was performed according to the following program: denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 60 seconds; 30 cycles of PCR were conducted. The PCR product was visualized by electrophoresis on 1% agarose gel.

3.2. Cloning of Keratinocyte Growth Factor Gene in pET28a

The PCR product and pET28a(+) expression vector were digested by NcoI and XhoI restriction enzymes (Fermentas, Lithuania). Purifications of the digested PCR products and vector from agarose gels were performed by agarose gel extraction kit (Bioneer, USA). The purified DNA fragment was cloned into pET28a(+) expression vector by T4 DNA ligase (Fermentas, Lithuania) and the ligated products were transformed into *E. coli* TOP10F′. Screening was performed by colony PCR. The recombinant plasmids were confirmed by restriction digestion.

3.3. Protein Expression

E. coli BL21(DE3) cells were transformed with recombinant vectors and grown at 37°C in luria broth (LB) medium containing appropriate antibiotic until the optical density at 600 nm of 0.4-0.6 was reached. Afterwards, 0.5 mM IPTG was added to the bacterial cultures to induce the expression of recombinant protein and they were incubated for 4 hours. Cellular pellets were collected by centrifugation at 6000 rpm for 5 minutes. First, total RNA was extracted and expression of recombinant protein was analyzed at RNA level by RT PCR. Then, expression was analyzed at protein level by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), so that 15 μL of each sample was loaded on 15% SDS-PAGE gel. After the electrophoresis, the gel was stained with coomassie blue.

3.4. Immunoblotting of the Recombinant Protein

The proteins were transformed from SDS-PAGE gel to a nitrocellulose membrane, using transfer buffer and electrophoresis. Afterwards, the nitrocellulose membrane was blocked with tween 20 at room temperature (RT) for two hours. The membrane was then washed three times by PBS-T and incubated in diluted (1:200) His-tag antibody (Abcam, USA) at RT for two hours. It was repeatedly washed prior to adding the diluted (1:2000) secondary antibody (HRP-conjugated, Abcam, USA) and then incubated at RT for one hour. The final reaction was detected using diaminobenzidine tetrahydrochloride (DAB; Sigma, USA) substrate.

3.5. Optimization of Expression

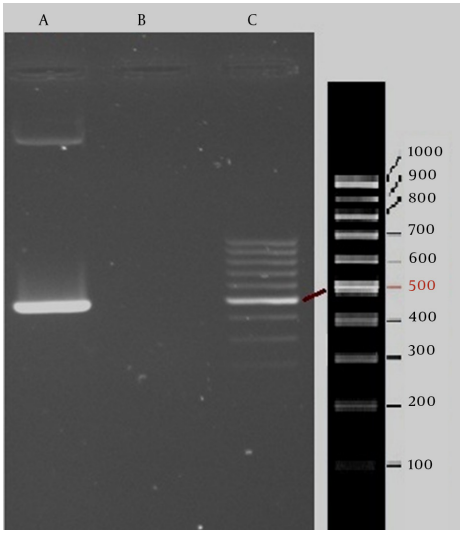
Optimization of recombinant protein expression was performed by changing various parameters such as harvesting time following IPTG induction and concentration of IPTG, to find the optimal conditions for expression of the recombinant protein. Different IPTG concentrations (0.1-1 mM) and harvesting times after induction (4, 8, 12 and 16 hours) were examined.

4. Results

4.1. Electrophoresis of the PCR Product

The *KGF* gene was amplified using designed primers by PCR and visualized on a 1% agarose gel (Figure 1).

Figure 1. Electrophoresis on 1 % Agarose Gel



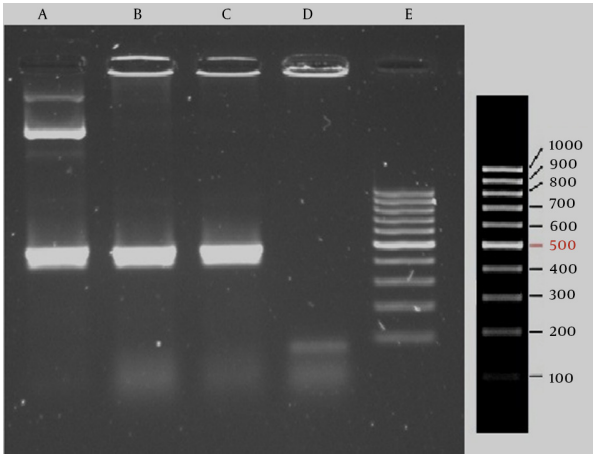
Lane A, PCR product of the *KGF* gene amplification; lane B, negative control; lane C, 100 bp DNA size marker (Fermentas, Lithuania).

4.2. Gene Cloning

The cloned *KGF* gene in pET28a was screened by colony PCR; a 497-bp product was detected, which was compatible with the size of *KGF* gene (Figure 2).

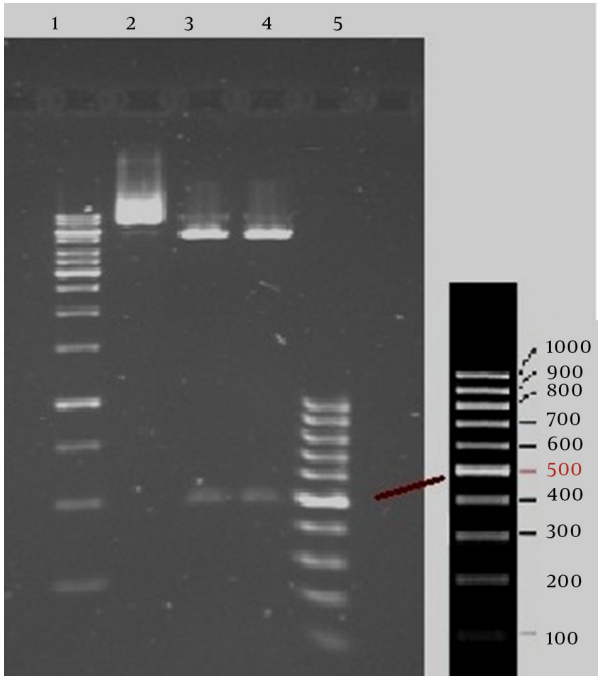
NcoI and XhoI restriction enzymes were applied to digest the plasmids extracted from positive colonies and presence of the 497-bp fragment confirmed cloning of the *KGF* gene into pET28a(+) vector (Figure 3).

Figure 2. Colony PCR



Lane A, positive control; lane B, The PCR-positive clone; lane C, The PCR-positive clone; lane D, negative control; lane E, 100-bp DNA size marker (Fermentas, Lithuania).

Figure 3. Double-Digestion of the Recombinant pET28a(+) Vector With XhoI and NcoI



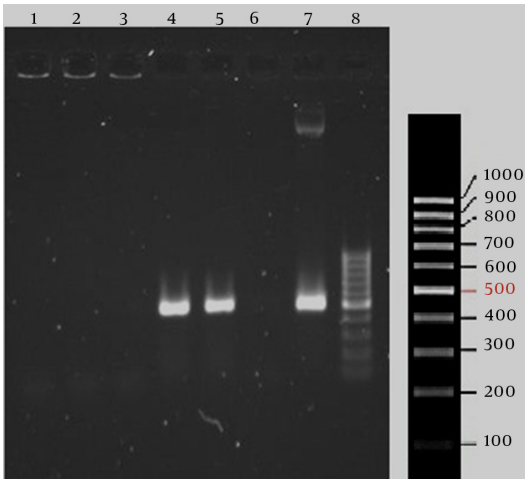
Lane 1, 1-kb DNA size marker (Fermentas, Lithuania); lane 2, undigested recombinant plasmid; lanes 3 and 4, digested recombinant plasmids; lane 5, 100-bp DNA size marker.

4.3. Protein Expression in Escherichia coli

A recombinant vector pET28a-*KGF* was transformed into *E. coli* BL21(DE3) and expression of the desired protein was investigated by IPTG induction at RNA and protein levels by RT-PCR and SDS-PAGE. The results of RT-PCR are shown in Figure 4.

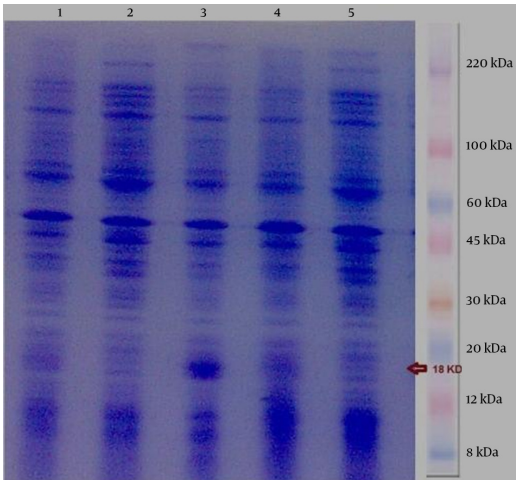
The results of SDS PAGE showed that the highest concentration of protein expression was at 0.3 mM IPTG and 16 hours after induction and a band with corresponding molecular weight marker of 18 kDa was identified. KGF protein expression in a concentration of 0.3 mM IPTG at various induction times were analyzed by SDS-PAGE, as shown in Figure 5.

Figure 4. RT-PCR



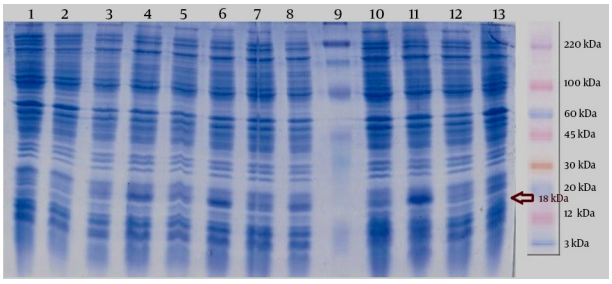
Lane 1, *E. coli* BL21 containing pET28a; lane 2, *E. coli* BL21 containing pET28a-*KGF* before induction; lane 3, RT-PCR negative control; lanes 4 and 5, *E. coli* BL21 containing pET28a-*KGF* after induction; lane 6, PCR negative control; lane 7, PCR positive control; lane 8, 100-bp DNA size marker.

Figure 5. Analysis of KGF Protein Expression at Different Harvesting Times in 0.3 mM IPTG Concentration



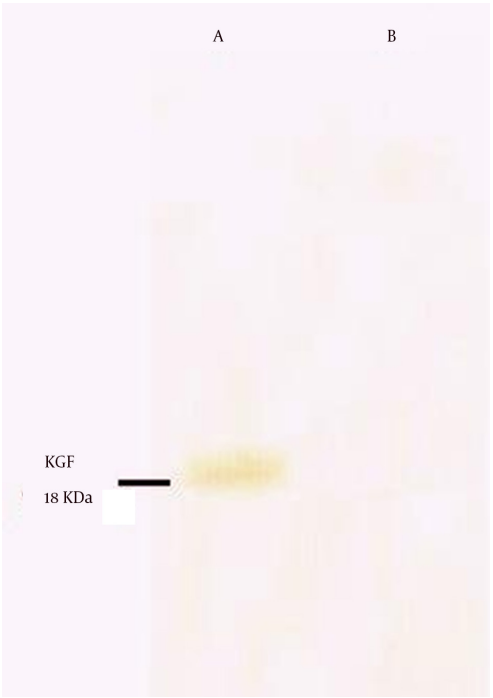
Lane 1, *E. coli* BL21 cell lysate with empty vector vector; lane 2, *E. coli* BL21 cell lysate transformed by recombinant plasmid (before induction); lane 3, *E. coli* BL21 cell lysate transformed by recombinant plasmid (16 hours after induction); lane 4, *E. coli* BL21 cell lysate transformed by recombinant plasmid (12 hours after induction); lane 5, *E. coli* BL21 cell lysate transformed by recombinant plasmid (8 hours after induction).

Figure 6. Analysis of KGF Protein Expression at Different Harvesting Times and Concentrations of IPTG



Lanes 1 and 2: *E. coli* BL21 cell lysate with empty vector (before and after IPTG induction). Lanes 3-8: *E. coli* BL21 cell lysate with recombinant plasmid before and after induction(4 h) by 1, 0.75 and 0.5 mM IPTG, respectively ; Lane 9: Protein size marker (Sigma, USA); Lanes 10-13: *E. coli* BL21 cell lysate with recombinant plasmid before and after induction(16 h) by 0.3 and 0.1 mM IPTG, respectively.

Figure 7. Western Blotting Analysis of Expressed r-KGF Using His-Tag Antibody



Lane A, *E. coli* BL21 cell lysate transformed by recombinant plasmid (after induction); lane B, *E. coli* BL21 cell lysate transformed by recombinant plasmid (before induction).

Immunoblotting with His-tag antibody confirmed the identity of the expressed recombinant protein (Figure 7).

5. Discussion

Chemotherapy and radiotherapy are the main ways to treat blood cancers. Oral mucositis is a common and often debilitating complication in cancer treatment (11, 12). Oral mucositis refers to the particular inflammation and ulceration after injury to epithelial cells that line the oral cavity. The damage causes changes ranging from mild atrophy to severe ulceration. Serious consequences include pain requiring analgesic drugs. Currently, there is no standard therapy for oral mucositis (13, 14). Therefore, identifying effective treatments for this condition is one of the useful measures. In this regard, beneficial therapeutic effects of KGF have been evaluated. Due to protective and regenerative effects of KGF on epithelial cells, morphogenesis of epithelium, and re-epithelialization of wounds, this factor plays a potential therapeutic role in treatment of oral mucositis. For overexpression of recombinant KGF protein, pET expression systems such as pET8c, pET9c and pET3c vectors were used in different studies. In this study, pET28a(+) was applied and good yields of recombinant protein production were observed. Because of its strong and specific promoter, pET system is the most powerful system yet developed for cloning and expression of recombinant proteins in *E. coli*.

Since nonglycosylated KGF has specific activity and due to advantages of *E. coli* expression system, it could be a suitable host for expression of recombinant human KGF. The host used in this study was *E. coli* Bl21(DE3). This bacterium has also been used in other researches (15, 16). This bacterial strain has major features; the most important feature is its small number of extracellular proteases. Therefore, protease activity on the expressed recombinant protein is less, compared with other bacterial or yeast hosts. Induction of lac promoter was performed using IPTG. In this study, by changing various parameters such as the harvesting time and concentration of IPTG, optimal conditions for protein expression were examined. Regarding the time of harvesting, the most appropriate time for expression was 16 hours after induction. To determine the appropriate concentration of IPTG, different concentrations were examined and 0.3 mM IPTG was the most suitable concentration to express the recombinant protein. At higher concentrations, expression of the protein was decreased. Results of the current study showed that induction of the recombinant clone pET28a-*KGF* in *E. coli* Bl21 by IPTG could produce recombinant protein in the host. At the next stage, for final confirmation of the expressed proteins, western blotting with a standard anti-His antibody was performed and the result was positive for recombinant KGF.

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Author's Contributions

Study concept and design: Massoud Saidijam; acquisition of data: Fatemeh Ebrahimzadeh and Ghasem Barati; analysis and interpretation of data: Hassan Mirza Hosseini and Massoud Saidijam; drafting of the manuscript: Fatemeh Ebrahimzadeh; critical revision of the manu-

script for important intellectual content: Massoud Saidijam; statistical analysis: Massoud Saidijam; administrative, technical, and material support: Yeganeh Talebkhan; study supervision: Massoud Saidijam.

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References

1. Beaven AW, Shea TC. Recombinant human keratinocyte growth factor palifermin reduces oral mucositis and improves patient outcomes after stem cell transplant. *Drugs Today (Barc).* 2007;**43**(7):461–73.
2. Radtke ML, Kolesar JM. Palifermin (Kepivance) for the treatment of oral mucositis in patients with hematologic malignancies requiring hematopoietic stem cell support. *J Oncol Pharm Pract.* 2005;**11**(3):121–5.
3. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc Natl Acad Sci U S A.* 1989;**86**(3):802–6.
4. Danilenko DM. Preclinical and early clinical development of keratinocyte growth factor, an epithelial-specific tissue growth factor. *Toxicol Pathol.* 1999;**27**(1):64–71.
5. Tsuboi R, Sato C, Kurita Y, Ron D, Rubin JS, Ogawa H. Keratinocyte growth factor (FGF-7) stimulates migration and plasminogen activator activity of normal human keratinocytes. *J Invest Dermatol.* 1993;**101**(1):49–53.
6. Marchese C, Rubin J, Ron D, Faggioni A, Torrisi MR, Messina A, et al. Human keratinocyte growth factor activity on proliferation and differentiation of human keratinocytes: differentiation response distinguishes KGF from EGF family. *J Cell Physiol.* 1990;**144**(2):326–32.
7. Werner S, Smola H, Liao X, Longaker MT, Krieg T, Hofschneider PH, et al. The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science.* 1994;**266**(5186):819–22.
8. Wildhaber BE, Yang H, Teitelbaum DH. Total parenteral nutrition-induced apoptosis in mouse intestinal epithelium: modulation by keratinocyte growth factor. *J Surg Res.* 2003;**112**(2):144–51.
9. Finch PW, Rubin JS. Keratinocyte growth factor/fibroblast growth factor 7, a homeostatic factor with therapeutic potential for epithelial protection and repair. *Adv Cancer Res.* 2004;**91**:69–136.
10. Werner S. Keratinocyte growth factor: a unique player in epithelial repair processes. *Cytokine Growth Factor Rev.* 1998;**9**(2):153–65.
11. McGuire DB, Altomonte V, Peterson DE, Wingard JR, Jones RJ, Grochow LB. Patterns of mucositis and pain in patients receiving preparative chemotherapy and bone marrow transplantation. *Oncol Nurs Forum.* 1993;**20**(10):1493–502.
12. Woo SB, Sonis ST, Monopoli MM, Sonis AL. A longitudinal study of oral ulcerative mucositis in bone marrow transplant recipients. *Cancer.* 1993;**72**(5):1612–7.
13. Armstrong TS. Stomatitis in the bone marrow transplant patient. An overview and proposed oral care protocol. *Cancer Nurs.* 1994;**17**(5):403–10.
14. Donnelly JP, Blijlevens NM, Verhagen CA. Can anything be done about oral mucositis? *Ann Oncol.* 2003;**14**(4):505–7.
15. Luo Y, Cho HH, Jones RB, Jin C, McKeehan WL. Improved production of recombinant fibroblast growth factor 7 (FGF7/KGF) from bacteria in high magnesium chloride. *Protein Expr Purif.* 2004;**33**(2):326–31.
16. Ron D, Bottaro DP, Finch PW, Morris D, Rubin JS, Aaronson SA. Expression of biologically active recombinant keratinocyte growth factor. Structure/function analysis of amino-terminal truncation mutants. *J Biol Chem.* 1993;**268**(4):2984–8.