ISSN : 1225-9918
Journal of Life Science **2011** Vol. 21. No. 6. 907~911 DOI : 10.5352/JLS.2011.21.6.907

- Note -

Production of Recombinant Human Keratinocyte Growth Factor from *Bombyx mori* (Lepidopera: Bombycidae) Bm5 Cells

Song-Yi Han[1], Cho-Yi Jin[1], Kisang Kwon[1], Eun Young Yun[2], Tae Won Goo[2], Seung-Whan Kim[3], Jong-Soon Choi[4,5], Kweon Yu[6] and O-Yu Kwon[1]*

[1]*Department of Anatomy, School of Medicine, Chungnam National University, Taejon 301-747, Korea*
[2]*Department of Sericulture & Entomology, National Institute of Agriculture Science and Technology, R.D.A., Suwon 441-100, Korea*
[3]*Department of Emergency Medicine, Chungnam National University Hospital, Taejon 301-721, Korea*
[4]*Division of Life Science, Korea Basic Science Institute, Taejon 305-333, Korea*
[5]*Graduate School of Analytical Science and Technology, Chungnam National University, Taejon 660-758, Korea*
[6]*Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea*

Received March 3, 2011 / Accepted May 24, 2011

Using silkworm *Bombyx mori* Bm5 cells, we established a stable cell line expressing the human keratinocyte growth factor (hKGF), named by the Bm5-hKGF cell, in which the protein hKGF is synthesized in the cell and secreted in the cell culture supernatant (CCS) at approximately 15-20 ng/ml. When the Bm5-hKGF cell was co-expressed with *B. mori* protein disulfide isomerase (bPDI) cDNA, its secretion increased by about two times the original amount. Through wound healing migration assay, it was demonstrated that the secreted hKGF included in the CCS has a very powerful biological activity of keratinocyte proliferation. We expect to produce useful human recombinant proteins from silkworm cultured cells in large quantities at low prices.

Key words : Silkworm *Bombyx mori* Bm5 cells, human keratinocyte growth factor, protein disulfide isomerase

Introduction

These days, many mammalian culture cell systems are used for production of recombinant proteins. However, expensive human hormones and fetal bovine/calf serum are required for maintenance of cell culture, which is one of the main factors in the increase of production-cost. In addition, those added hormones create two major problems that not only cause difficulty in purification of recombinant proteins, but also production of different components by each process. Recently, insect cell culture systems have been strongly considered as bioreactors instead of mammalian culture cell systems, because insect cells have mammalian cell-like endoplasmic reticulum for production of glycoprotein. Actually, the insect cell of the silkworm *Bombyx mori,* Bm5, has an endoplasmic reticulum in which mammalian-like post-translational modification processes occur, giving it merit as a potentially valuable tool for mass production of low-cost glycoprotein using no expensive hormones and/or growth factors. So far, although many types of recombinant proteins are successfully produced by a baculovirus-expression system using Bm5 cells, direct production of useful those from Bm5 cells has some advantages, including no baculovirus inactivation step, and no rejection of consumers of useful biomaterials produced by baculovirus [5].

Keratinocyte growth factor (KGF) is encoded by the *FGF7* gene, and is a member of the fibroblast growth factor family, which shows broad biological activities. Many recent studies of the biological/pharmacological function of KGF have reported results that can be directly applied for clinical use. For example, stimulation of macrophage inflammatory protein 3 alpha and keratinocyte-derived chemokine secretion, prevention of intestinal mucositis by KGF, better repair of skin defects and radiation-induced wounds, selective protection of normal epithelia during radiotherapy, and combined-treatment of KGF with p53 improve T-cell reconstitution after bone marrow transplantation [1,4,6,8]. Following a number of clinical demands, recombinant human KGF (hKGF) has been produced in many organisms, including microbes, yeast, mammalian cells, and plant cells. However, these organisms have weak points for production of bioactive type recombinant hKGF. Here, for the first time,

***Corresponding author**
Tel : +82-42-580-8206, Fax : +82-42-586-4800
E-mail : oykwon@cnu.ac.kr

we have established a transgenic Bm5 (Bm5- hKGF) cell line in which constantly bioactive recombinant hKGF was secreted, with results showing remarkable proliferation of human keratinocyte cells. In addition, we obtained results showing that *B. mori* protein disulfide isomerase (bPDI) increased its production about two times. We suggest that the protein production system using insect cells of silkworm *B. mori* can be expected to supply recombinant proteins on a large scale at a low price.

Materials and Methods

Bm5 cells (silkworm *Bombyx mori* cell line derived from ovary) were cultured in Grace's insect medium (WelGENE, Daegu, Korea), including 10% FBS and 1% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, USA), at 26℃ [2]. A cDNA encoding hKGF was cloned into the *Sac* I and *EcoR* V sites of the insect expression vector pIZT/V5-His (containing green fluorescent protein as a screening marker). The resulting vector was transfected into Bm5 cells using lipofectin reagent (Invitrogen, Carlsbad, USA). Transfected cells were cultured under 200 μg/ml antibiotic Zeocin (Invitrogen) for 14 day. Successfully transfected cells (Bm5-hGM-CSF) were screened by RT-PCR and green fluorescence under a fluorescence microscope. Total RNA from cultured cells was extracted using an RNA isolation reagent (TRI-Reagent; Ambion, Austin, USA). RT-PCR using the forward primer (F) 5′-TATATAGAGCTCATGCCGC TGGAG-3′ and the reverse primer (R) 5′-GGGGGGGTCT AGATAAGACTTGATTTTT-3′ for hKGF was performed for 27 cycles with the follows conditions: 94℃ for 30 sec; 57℃ for 40 sec; and 72℃ for 40 sec (but 10 min in the final cycle) with *Taq* DNA polymerase. Immunoblotting analysis was performed according to standard procedures. Bm5-hKGF cells were scraped, lysed by addition of SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% glycerol, 125 mM DTT, 0.03% (w/v) bromophenol blue], and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, which was then incubated with the primary antibody (recognizing His tag) overnight at 4℃. Blots were developed using the kit for the ECL Western blotting detection system (GE Healthcare, Piscataway, USA) and BioMax Light film (Kodak, Rochester, USA). ELISA (enzyme-linked immunosorbent assay) was performed using the hKGF ELISA kit (KOMA Biotech, Seoul, Korea) in 96-well culture plates. After treatment with the coating antibody recognized by hKGF for 2 hr at room temperature, cell culture supernatant (CCS) containing secreted hKGF was added and reacted. After 2 hr at room temperature and washing with PBST (0.1% Tween 20 in PBS), horseradish-peroxidase (30 min reaction) and TMB solution (10 min reaction) were added. When light was emitted, 2 M H_2SO_4 was added, and the 96-well culture plate was estimated at 450 nm by a microplate reader. Wound healing migration assay was performed as described with modification [7]. Human keratinocytes of HaCaT were cultured to confluence in a 6-well plate. The monolayer culture was then scrape-wounded with a sterile micropipette tip to create a denuded gap of constant width. After washing with PBS for removal of cellular debris, cells were exposed to various conditions. The scratch on the monolayer culture was observed by inverted microscope and photographed immediately after wounding for 24 hr. The wound area was measured by the program Image J. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich.

Results and Discussion

Bm5 cells were transfected with the modified pIZT/V5-His vector containing genes of both green fluorescent protein (GFP) and hKGF, for easy observation of expression of the transfectant using a fluorescence microscope. For selection of cells with stable expression of the hKGF gene, Zeocin was added at a concentration of 200 μg/ml 12 hr post-transfection. GFP was observed 5 day after transfection. By that time, Bm5 control cells were detached from the culture plate. After 14 day of transfection, a transfectant containing hKGF, coined as Bm5-hKGF cells, was confirmed by Western blot analysis using His 1^{st} antibody.

Fig. 1A shows the results from Western blotting; on the left panel, hKGF was detected only in the cell lysate of Bm5-hKGF cells between 20-25 kDa, which means that a transfected cDNA of hKGF was successfully processed to transcription and translation in Bm5 cells. Next, we checked to see whether or not hKGF in a cell is correctly secreted into the extracellular environment. On the right panel, a 25 kDa hKGF was detected at cell culture supernatant (CCS) collection of Bm5-hKGF cells. Although many types of cDNA can be transfected into a cultured cell line, it is not always expressed by its mRNA and protein. These results demonstrated that exogenous hKGF cDNA was successfully transcripted and translated (left panel), and secreted (right

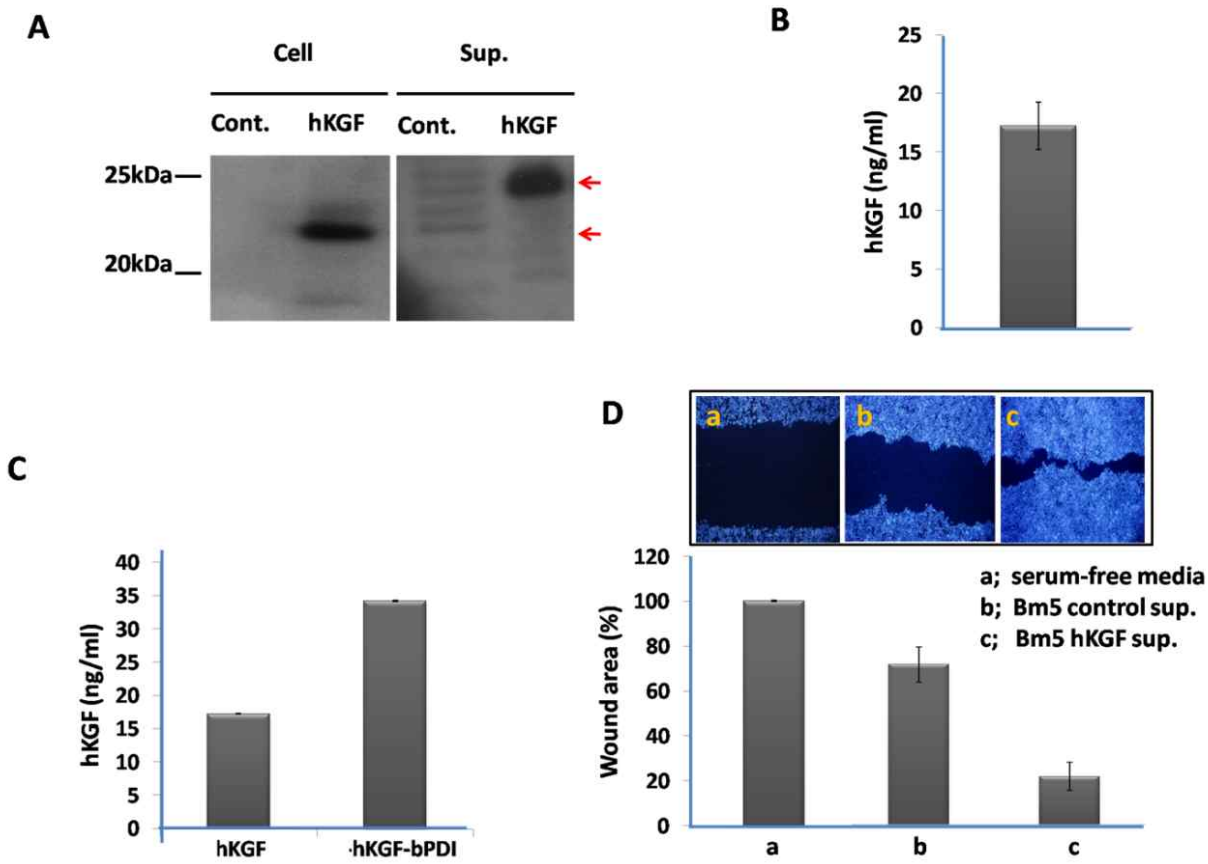

Fig. 1. Production of recombinant hKGF from *Bombyx mori* Bm5 cells. (A) Western blotting of hKGF. Bm5 cells were cultured in Grace's insect medium that included 10% FBS and 1% antibiotic-antimycotic solution at 26°C. Insect expression vector pIZT/V5-His contained hKGF cDNA in the *Kpn* I and *EcoR* I sites. The resulting vector was transfected into Bm5 cells using lipofectin, and transfected cells (Bm5-hKGF cells) were screened under Zeocin (200 μg/ml) for 14 day. The RT-PCR product of hKGF corresponding to the full length hKGF was detected (data not shown). Left panel is the cell lysate. Recombinant hKGF synthesized intracellularly is indicated by a lower arrow. Right panel is cell culture supernatant (CCS) collection from Bm5-hKGF cells; a 25 kDa hKGF is indicated by an upper arrow. At both panels, each left lane is the negative control using non-transfected cells. (B) Determination of hKGF concentration in CSS by ELISA. ELISA assay processing was performed using the hKGF ELISA kit (KOMA Biotech, Korea), following the manufacturer's instructions. The final concentration was estimated at 450 nm by a microplate reader. (C) bPDI expression increased hKGF secretion from Bm5-hKGF cells. hKGF, secretory hKGF from Bm5-hKGF cells; hKGF-bPDI, secretory hKGF from Bm5-hKGF cells co-transfected with bPDI. hKGF concentration in CSS was measured by ELISA kit. (D) Wound healing migration assay. Monolayers of Bm5-hKGF cells were scraped by a sterile micropipette tip and the cells were treated with various concentrations. a, treated with serum free medium; b, normal culture medium; c, with CCS containing the secretory form of recombinant hKGF (50 ng/ml) for 24 hr. The wounded region was measured by the program Image J. The wound area of the culture was quantified in four fields in each treatment, and data were calculated from three independent experiments.

panel) from Bm5-hKGF cells. We think that the cause of increased molecular weight of hKGF (right panel) for the secreted form is due to posttranslational modification of glycosylation (at 45th-amino acid) through the endoplasmic reticulum quality control system.

The highest level of hKGF production in CCS collection of Bm5-hKGF cells was detected by Western blotting 48 hr following exchange of new culture medium. At this time, the concentration of hKGF in CCS was estimated by ELISA. The average concentration of secreted hKGF was 17.2 ng/ml (Fig. 1B). We have previously demonstrated that *B. mori* protein disulfide isomerase (bPDI) accelerated production of recombinant proteins in Bm5 cells [3]. To produce a lot of hKGF, a cDNA of bPDI was co-transfected in Bm5-hKGF

cells. As shown Fig. 1C, we obtained approximately 2 times the amount of the secreted form of hKGF (approximately 35 ng/ ml) compared with its control. In this case, no cell morphological change and no early cell detachment were observed (data not shown).

We next used the wound healing migration assay for determination of whether or not hKGF in CCS has bioactivity. The monolayer culture of human keratinocyte HaCaT was scrape-wounded using a tip to create a denuded zone of constant width. After exposure of the cells to different conditions for 48 hr, respectively, one of three groups was treated with CCS containing 50 ng hKGF (Fig. 1D-c). The other two groups were treated with Bm5 culture medium (Fig. 1D-b) and serum-free medium (Fig. 1D-a). As indicated in Fig. 1D, the wound healing ability of HaCaT cells treated with serum-free medium was markedly reduced, compared to that observed in cells treated with CCS containing 50 ng hKGF. We eventually demonstrated that hKGF produced from Bm5-hKGF cells showed increased growth of human keratinocytes nearly 5-fold higher than that of the control. In addition, no morphologically abnormal keratinocytes were detected (data not shown). It is then suggested that secreted recombinant hKGF from Bm5 cells has a valuable biological activity for keratinocyte proliferation.

In summary, one of the major problems in biotechnology of recombinant protein production is that secretory and membrane-bound proteins are generally produced in lower amounts in insect cells compared to cytoplasmic and nuclear proteins. In this study, exogenous human cDNA encoding hKGF was successfully biosynthesized through correct transcription and translation, eventually resulting in significant production of the secretory form of the bioactive protein in Bm5 cells (*B. mori* silkworm cell line). Other major advantages of using Bm5 cells include low cost, mass-production of bioactive proteins, and freedom from biohazards, with no need for expensive hormones for culture.

Acknowledgements

This work was supported by grants from the BioGreen 21 Program (20070401034024), Korea Science & Engineering Foundation (2008-0061669) and Korea Basic Science Institute (T30602).

References

1. Athar, U. and T. C. Gentile. 2009. Keratinocyte growth factor. *Expert Opin. Biol. Ther.* **9,** 779-787.
2. Goo, T. W., E. Y. Yun, K. H. Choi, S. H. Kim, S. K. Nho, S. W. Kang, and O. Y. Kwon. 2004. ATFC is a novel transducer for the unfolded protein response in Bombyx mori BM5 cells. *Biochem. Biophys. Res. Commun.* **325,** 626-631.
3. Goo, T. W., E. Y. Yun, S. W. Kim, K. H. Choi, S. W. Kang, K. Kwon, K. Yu, and O. Y. Kwon. 2008. *Bombyx mori* protein disulfide isomerase enhances the production of nuecin, an antibacterial protein. *BMB Reports* **41,** 400-403.
4. Hille, A., S. Grüger, H. Christiansen, H. A. Wolff, B. Volkmer, J. Lehmann, W. Dörr, and M. Rave-Fränk. 2010. Effect of tumour-cell-derived or recombinant keratinocyte growth factor (KGF) on proliferation and radioresponse of human epithelial tumour cells (HNSCC) and normal keratinocytes in vitro. *Radiat. Environ. Biophys.* **49,** 261-270.
5. Hitchman, R. B., R. D. Possee, and L. A. King. 2009. Baculovirus expression systems for recombinant protein production in insect cells. *Recent Pat. Biotechnol.* **3,** 46-54.
6. Kelly, R. M., E. M. Goren, P. A. Taylor, S. N. Mueller, H. E. Stefanski, M. J. Osborn, H. S. Scott, E. A. Komarova, A. V. Gudkov, G. A. Holländer, and B. R. Blazar. 2010. Short-term inhibition of p53 combined with keratinocyte growth factor improves thymic epithelial cell recovery and enhances T-cell reconstitution after murine bone marrow transplantation. *Blood* **115**, 1088-1097.
7. Rodriguez, L. G., X. Wu, and J. L. Guan. 2005. Wound-healing assay. *Methods Mol. Biol.* **294**, 23-29.
8. Ryu, S. H., S. Y. Moon, Y. J. Yang, S. R. Moon, J. P. Hong, J. Choi, and S. W. Lee. 2009. Recombinant human epidermal growth factor accelerates the proliferation of irradiated human fibroblasts and keratinocytes *in vitro* and *in vivo*. *J. Radiat. Res.* **50**, 545-552.

초록 : 누에배양세포에서 인간형 재조합단백질 각질세포 성장인자 생산

한송이[1] · 진초이[1] · 권기상[1] · 윤은영[2] · 구태원[2] · 김승환[3] · 최종순[4,5] · 유권[6] · 권오유[1]*

([1]충남대학교 의학전문대학원 해부학교실, [2]농촌진흥청 국립농업과학원, [3]충남대병원 응급의학과, [4]한국기초과학지원연구원 생명과학연구부, [5]충남대학교 분석과학기술대학원, [6]한국생명공학연구원 장수과학연구센터)

인간형 재조합단백질 각질세포 성장인자를 안정적으로 생산하는 누에 배양 세포(Bm5-hKGF cell)을 만들었다, 이 세포에서 분비되어 배지에 포함된 양은 15-20 ng/ml 정도였다. Bm5-hKGF cell에 누에의 PDI를 함께 발현시키면 세포외 분비량이 2배 증가하였다. Wound healing migration assay 결과 누에세포에서 생산된 인간형 재조합단백질 각질세포 성장인자는 세포생장을 촉진하는 활성을 가지고 있었다. 본 실험의 결과는 누에배양세포를 사용하여 저비용으로 양질의 인간형 재조합단백질을 대량생산 할 수 있는 것을 기대한다.