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Expression of Recombinant Human Epidermal Growth Factor in *Escherichia coli* and Characterization of its Biological Activity

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Abstract Recombinant human epidermal growth factor (EGF) was successfully expressed as a fusion protein in *Escherichia coli* system. This system was used OmpA signal sequence to produce soluble protein into the periplasm of *E. coli*. Human EGF (hEGF) synthesized in bacterial cell was found to be similar in size with the original protein and molecular weight approximately at 6.8 kDa. Cell proliferation assay was conducted to characterize the biological activity of hEGF on human dermal fibroblasts. The synthesized hEGF was found to be functional as compared with authentic hEGF in stimulating cell proliferation and promoting growth of cell. In comparison of biological activity between synthesized and commercial hEGF on cell proliferation, the results showed there was no significant different. This finding indicates the synthesized hEGF in *E. coli* system is fully bioactive in vitro.

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Abbreviations

hEGF	human epidermal growth factor
HDF	human dermal fibroblasts
IPTG	isopropylthiogalactopyranoside
EDTA	ethylenediaminetetraacetic acid
LB	Luria Bertani
TBS	tris buffer saline
NaCl	sodium chloride
Tris-HCl	tris-hydrochloride
MTT	methylthiazolyldiphenyl-tetrazolium bromide
E. coli	*Escherichia coli*
PBS	phosphate buffer saline
CO_2	carbon dioxide

Introduction

The *Escherichia coli* expression system is known to be the fastest, easiest, and an inexpensive technique used to express usable amounts of recombinant protein. These characteristics, coupled with advance knowledge about *E. coli* and the many years' experience with expression of foreign genes, have established *E. coli* as the leading host organism for most scientific applications in protein expression [1].

Epidermal growth factor (EGF) is a single-chain polypeptide consisting of 53 amino acids with molecular weight of about 6,200 Da. The six cysteine residues in the sequence of hEGF formed three disulfide bonds, which are required for human EGF (hEGF) to be biologically active [2]. EGF was first isolated from the submaxillary glands of adult male mice [3] and discovered in human urine as an inhibitor of gastric acid secretion [4]. Later, it was proved that EGF caused both proliferation of epithelial cells and inhibition of gastric acid secretion.

Now, hEGF has become one of the most popular growth factors studied in life science as well as biotechnological fields. hEGF is a potent stimulator of epithelial cell, endothelial cell, and fibroblast proliferation both in vitro and in vivo, which results in its potential as a promising healing agent for the treatment of various skin and corneal wounds [5]. It is also a good candidate for the treatment of gastric ulcers because it inhibits gastric acid secretion and regenerates gastric mucosal layer [4].

Previous studies had attempted to identify the methods of achieving sufficient quantities of hEGF to conduct the extensive studies on its biological activity and clinical applications [6]. It is desirable that recombinant products to be produced in *E. coli* is secreted into the periplasmic space or cell growth medium. Purification would then be simpler for an intracellular protein as the product would not be contaminated with cytoplasmic components [7].

In addition, the formation of inclusion bodies would be avoided, and possible toxic effects of the hEGF polypeptide product on the host cell would be reduced [8]. To solve these problems, an ampicillin (Amp)-resistant *E. coli* strain DH5α was used to produce secreted hEGF protein. A synthetic gene for hEGF was fused with the Outer membrane protein A (OmpA) signal peptide that was contained in the pFLAG-ATS Expression Vector (Sigma Aldrich, USA). The OmpA signal sequence is responsible for the secretion of OmpA, an abundant protein in *E. coli* from cytoplasmic space into the periplasmic space

that considered free of inclusion bodies and no possible toxic effects on the host cell. The excreted proteins known as fusion proteins are usually stable, not significantly degraded, and have low levels of extracellular protease activity.

Eventually, the study on the biological activity of hEGF expressed in *E. coli* needs to be conducted, because it is very important to verify that the synthesized hEGF was bioactive and completely identical to the original protein and standard hEGF.

Materials and Methods

Expression and Isolation of hEGF

A recombinant plasmid, pFLAG-ATS (hEGF) was constructed and transformed into *E. coli* strain, DH5α [9]. An individual colony was picked from Luria Bertani (LB) agar plate and inoculated into 5 ml LB medium containing 50 μg/ml Amp and 0.4% glucose and then incubated in the orbital shaker overnight at 37°C. The following day, 1:100 of 5 ml culture was inoculated into 100 ml LB medium containing 50 μg/ml Amp and 0.4% glucose and grown at 37°C to Optical Density $(OD)_{600}$ of 0.5–0.8. The cells were then induced with 1.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG; Eppendorf, Germany) grown for additional 2 h at 37°C and pelleted by centrifugation at 5,000×*g* for 10 min. The pellet was warmed to room temperature and resuspended in 40 ml/g cells of 0.5 M Sucrose, 0.03 M Tris, and 1 mM ethylenediamine tetraacetic acid (EDTA) at a final pH of 8.0 per gram of cells. The mixture was then centrifuged at 3,500×*g* for 10 min at 10°C for osmotic shock procedure to extract the hEGF protein in a periplasmic fraction. The supernatant was discarded, and the pellet was rapidly resuspended in 25 ml/g cells of ice-cold distilled water. The mixture was then centrifuged at 3,500×*g* for 10 min at 4°C, and the supernatant was collected immediately for periplasmic hEGF protein.

Purification of hEGF with ANTI-FLAG M2 Affinity Gel

Recombinant hEGF was purified using FLAG Fusion Protein Immunoprecipitation Kit (Sigma Aldrich, USA). This kit provides a rapid and efficient immunoprecipitation and elution of an active FLAG-tagged protein. The procedure includes several steps of centrifugation and supernatant removal. The ANTI-FLAG M2 affinity gel resin was suspended with tris buffer saline [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] at a ratio of 2:1. Immediately, 40 μl of the resin in its suspension buffer was transferred to a fresh test tube to allow a homogenous dispensation of the resin. The resin was then centrifuged for 30 s at 5,000×*g*, and the supernatant was removed with a narrow-end pipette tip. After that, the resin was washed 4–5 times using 0.5 ml of 1× Wash Buffer for each wash. A 1,000-μl crude protein was added to the washed resin. All samples were shaken gently overnight. After overnight incubation, the resin was centrifuged for 30 s at 5,000×*g*, and the supernatant was removed with a narrow-end pipette tip. The resin was then washed three times with 0.5 ml of 1× Wash Buffer, and all supernatant was removed.

Elution of the hEGF Protein

hEGF protein was eluted under native condition by a competition with 3X FLAG peptide (Sigma Aldrich, USA). The elution efficiency is very high using this method. A 100-μl 3X FLAG Elution Buffer (Sigma Aldrich, USA) was added to the resin in the test tube and

incubated with gentle shaking for 30 min at 4°C. The mixture was then centrifuged for 30 s at 5,000×*g*, and the supernatant was transferred to fresh test tubes using a narrow-end pipette tip. The supernatant, which contained FLAG-fusion protein. was stored at −20°C before precipitation.

Removal of the FLAG Marker

The low-molecular-weight hydrophilic FLAG marker should not interfere with the biological activity of the native protein. The removal of the FLAG peptide was done by adding 1.0 unit of enterokinase per μg of FLAG fusion protein. Digestion was carried out in Enterokinase Reaction Buffer (Sigma Aldrich, USA), and the digestion mixture was incubated at 37°C for 18 h using PTC-100 Thermacycler (MJ Research).

Quantification of hEGF Protein by Sandwich ELISA

Concentration of hEGF protein was quantified using EGF, hEGF, BioAssay™ ELISA Kit (United States Biological, USA) according to the recommendation. Serial dilutions of EGF standard in the range of 31.3 to 1,000 pg/ml were prepared, and the amount of hEGF protein in the samples was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Sunrise, Tecan, Austria). All measurements were performed in duplicate, and the results were calculated as an average of two readings (kit recommendation).

Immunoblot Analysis of the hEGF Protein

The hEGF protein was first resolved on a 15% Tris-glycine sodium dodecyl sulfate (SDS)-polyacrylamide gel, then transferred onto a polyvinylidene difluoride membrane (Millipore, USA) and subsequently reacted with a mouse anti-hEGF monoclonal antibody (Abcam, USA). Monoclonal anti-hEGF (2.0 mg/ml) with 1:1,000 dilutions was used as primary antibody and anti-mouse IgG labeled by horseradish peroxidase (1:1,000 dilutions) as secondary antibody. Binding of the secondary antibody was developed using DAB substrate (Chemicon, USA) color reagents.

Human Dermal Fibroblasts

Normal Human Dermal Fibroblasts (HDF) from Cell Applications, Inc. was grown in fibroblasts growth media (with serum) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were trypsinized at room temperature when they reached confluency using Trypsin/EDTA Solution and Trypsin Neutralizing solution (Cell Applications, Inc).

MTT Cell Proliferation Assay on HDF

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was used to characterize the bioactivity of hEGF expressed in *E. coli* on monolayer cultures of HDF. The synthesized hEGF and standard hEGF were serially diluted (tenfold steps) from 10 ng/ml to 1 pg/ml with the basal medium, and 100 μl was added to each well. HDF at a density of 1×10^4 cells/ml was prepared in basal media and seeded 100 μl/well on sterile 96-well tissue culture plate. Incubation at 37°C and 5% CO_2 for 24, 48, and 72 h was done; after this was an addition of 10 μl/well MTT solution dissolved in PBS. The plates were incubated for an additional 4 h

at 37°C and 5% CO_2. Subsequently, 50 μl/well of dimethyl sulfoxide were added, and the plates were further incubated at 37°C and 5% CO_2 for an additional 30 min After the incubation, absorbance was measured at 570 nm using an ELISA reader (Sunrise, Tecan). The bioactivity curve of corrected absorbance values 570 nm on Y-axis and concentration of growth factor on X-axis was plotted.

Percentage of Growth

The percentage of growth of human embryonic kidney (HEK) was calculated using the formula:

$$\text{Percentage of growth, } \% = \frac{\text{Absorbance sample} - \text{Absorbance control}}{\text{Absorbance control}} \times 100\%$$

Statistical Analysis

t test was used to compare the proliferative effects of different concentrations of periplasmic hEGF and standard hEGF on HEK. These results were representatives of three independent experiments. The findings were considered significant when p value<0.05.

Results

Immunoblot Analysis of the hEGF Protein

The hEGF protein was synthesized in *E. coli* as a FLAG fusion protein with a molecular weight of approximately 6.8 kDa. FLAG tag was cleaved and removed from the fusion protein to release the authentic hEGF protein. Figures 1 and 2 showed the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of the purified hEGF protein expressed from *E. coli* in a periplasmic fraction. The purified hEGF protein (lane 3) demonstrated single band on SDS-PAGE stained with Bio-Safe™ Coomassie Blue (Bio-Rad, USA; Fig. 1). It also reacted strongly to the anti-hEGF monoclonal antibody, which visualized as a distinct thick band on the polyvinylidene difluoride membrane (lane 3) as compared to the standard hEGF (lane 2) in Fig. 2.

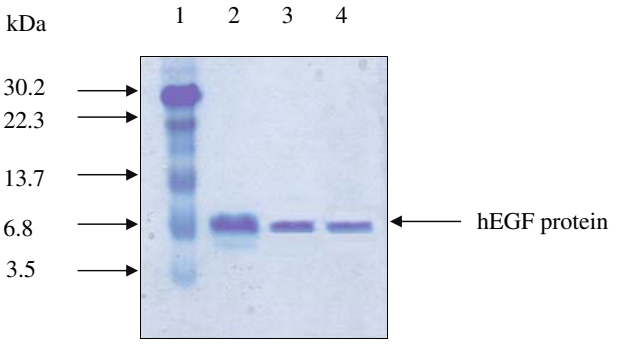

Fig. 1 SDS-PAGE analysis of purified hEGF protein, which expressed as periplasmic fraction in *Escherichia coli*. *Lane 1* shows Kaleidoscope Polypeptide Standard (Bio-Rad, U.S.A.). *Lane 2* shows Standard hEGF (Bio-Source, U.S.A.). *Lane 3* shows periplasmic fraction. *Lane 4* shows cytoplasmic fraction

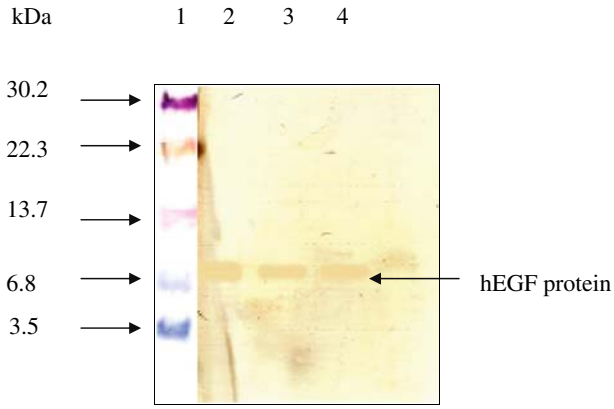

Fig. 2 Western blot analysis of purified hEGF protein using monoclonal anti-hEGF produced in mouse (Abcam, USA). *Lane 1* shows Kaleidoscope Polypeptide Standard (Bio-Rad, USA). *Lane 2* shows Standard hEGF (Bio-Source, USA). *Lane 3* shows periplasmic fraction. *Lane 4* shows cytoplasmic fraction

Quantification of hEGF Protein by Sandwich ELISA

Sandwich ELISA was used to quantify the hEGF protein expressed as periplasmic fraction in *E. coli*. Table 1 showed the concentration of hEGF protein expressed as periplasmic and cytoplasmic fractions with induction of 1.0 mM IPTG.

MTT Cell Proliferation Assay on HDF

MTT Cell Proliferation Assay is a colorimetric assay system which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. After incubation of the cells with the MTT reagent for approximately 2 h, a detergent solution was added to lyse the cells and solubilize the colored crystals. The samples were measured at 570 nm, and the proliferation effect of standard hEGF and periplasmic hEGF on the cells was calculated and plotted against the cells treated with media alone. Each point and vertical line plotted showed the mean value and the standard deviation of triplicates.

Proliferative Effect at 24, 48, and 72 Hour Incubation

The proliferative effect of periplasmic hEGF at different concentrations on HDF after 24 h incubation is shown in Fig. 3. The proliferative effect was found to be significant ($p<0.05$) at concentration 10 ng/ml as compared with concentration 0 ng/ml with 1.2-fold increase, at 1 ng/ml, 0.1 ng/ml, and 0.01 ng/ml with 1.1-fold increase, and at 0.001 ng/ml with 1.06-fold increase. A significant increase in the cell proliferation of HDF was observed with increasing concentrations of periplasmic hEGF. However, there was only significant difference of cell proliferation at 10 and 1 ng/ml as compared with 0.001 ng/ml. Incubation of periplasmic hEGF on HDF was extended to 48 h incubation (Fig. 4). At concentration 10 ng/ml, there was found a significant difference of proliferative effect as compared with

Table 1 Concentration of hEGF protein expressed as periplasmic and cytoplasmic fractions in *Escherichia coli* with induction of 1.0 mM IPTG.

Fraction	Periplasmic	Cytoplasmic
Incubation time, h	2	2
hEGF concentration, ng/ml	196.5	167

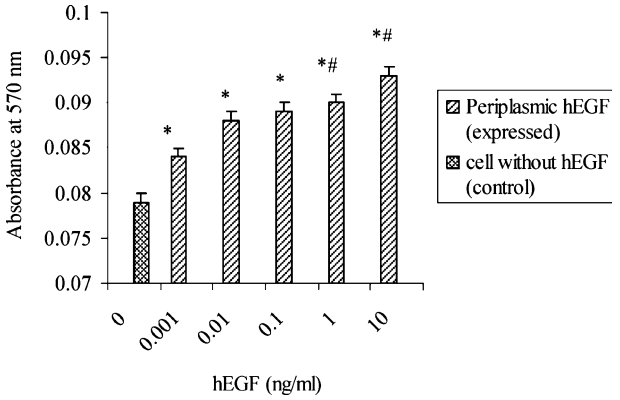

Fig. 3 Bioactivity assay of periplasmic hEGF (expressed) on HDF by using MTT colorimetric assay after 24 h incubation. *Asterisks* show a significant difference ($p<0.05$) from the corresponding control (no addition of periplasmic hEGF). *Hatch keys* show a significant difference ($p<0.05$) compared with 0.001 ng/ml periplasmic hEGF concentration

concentration 0 ng/ml with 1.35-fold increase, 1 ng/ml with 1.32-fold increase, 0.1 ng/ml with 1.32-fold increase, 0.01 ng/ml with 1.26-fold increase, and 0.001 ng/ml with 1.2-fold increase. Significant difference of cell proliferation was observed at 10 and 1 ng/ml as compared with 0.01 and 0.001 ng/ml periplasmic hEGF concentrations. Similar findings as that at 24- and 48-h incubation were observed even when the incubation period was extended to 72 h (Fig. 5). The proliferative effect of periplasmic hEGF was found to be significantly different at concentration 10 ng/ml as compared with the concentration of 0 ng/ml with 1.61-fold increase, which further increased at 1 ng/ml with 1.63-fold and eventually dropped at 0.1 ng/ml with 1.59-fold, 0.01 ng/ml with 1.57-fold, and 0.001 ng/ml with 1.5-fold. Significant difference of cell proliferation was also observed at 10, 1, 0.1, and 0.01 ng/ml as compared with 0.001-ng/ml periplasmic hEGF concentration.

Comparison Percentage of Growth of HDF After Incubation with Standard hEGF and Periplasmic hEGF

The comparison of the percentage of growth of HDF after 24, 48, and 72 h incubation with hEGF (commercial) and periplasmic hEGF (expressed) is shown in Fig. 6. No significant difference was observed between both proteins after the incubation time. Highest percentage of growth was found at concentration 10 ng/ml hEGF with 16.5% of 24 h incubation, 35% of 48 h incubation, and 64.8% of 72 h incubation. The result showed the percentage of growth was proportionally increased with increasing of growth factor concentrations. In addition,

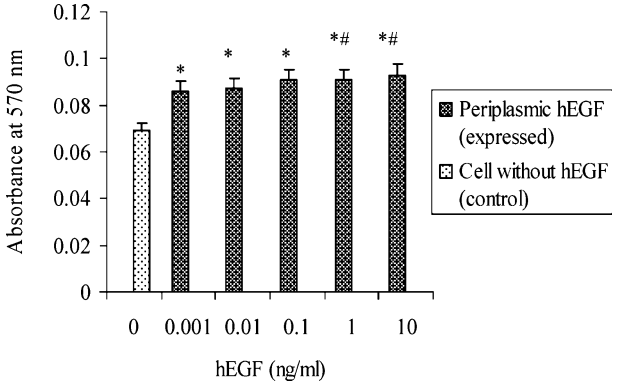

Fig. 4 Bioactivity assay of periplasmic hEGF (expressed) on HDF by using MTT colorimetric assay after 48 h incubation. *Asterisks* show a significant difference ($p<0.05$) from the corresponding control (no addition of periplasmic hEGF). *Hatch keys* show a significant difference ($p<0.05$) as compared with 0.01 and 0.001 ng/ml periplasmic hEGF concentrations

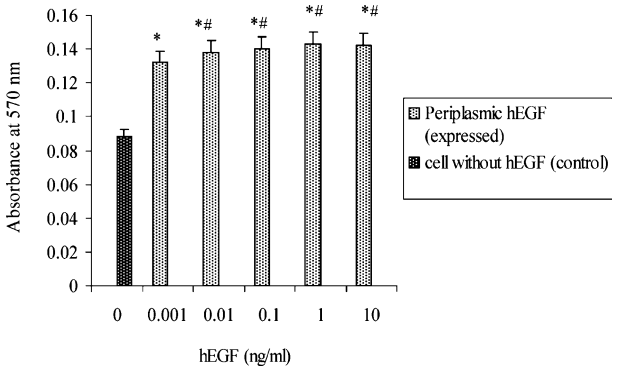

Fig. 5 Bioactivity assay of periplasmic hEGF (expressed) on HDF by using MTT colorimetric assay after 72 h incubation. *Asterisks* show a significant difference ($p<0.05$) from the corresponding control (no addition of periplasmic hEGF). *Hatch keys* show a significant difference ($p<0.05$) as compared with 0.001 ng/ml periplasmic hEGF concentration

there is an effective dose (EC_{50}) of hEGF concentration at 0.001 ng/ml on the growth of HDF after 72 h incubation. EC_{50} is determined as the hEGF concentration that is required to stimulate the growth of HDF to half that of control (cell without hEGF).

Tables 2 and 3 shows the percentage of growth of HDF incubated with hEGF (commercial) and periplasmic hEGF (expressed) at 24-, 48-, and 72-h incubations. At 24-h incubation, significantly higher percentage of growth of HDF was observed at concentrations 10 and 1 ng/ml as compared with 0.001 ng/ml. At 48-h incubation, percentage of HDF growth was found to be significantly different between hEGF concentrations at 10 and 1 ng/ml as compared with 0.01 and 0.001 ng/ml. Meanwhile, a significant increase of the percentage of growth was observed between hEGF concentrations at 10, 1, 0.1, and 0.01 ng/ml as compared with 0.001 ng/ml for 72-h incubation. In addition, the findings for each hEGF concentrations and the percentage of HDF growth were found to be significantly dependent on duration of incubation.

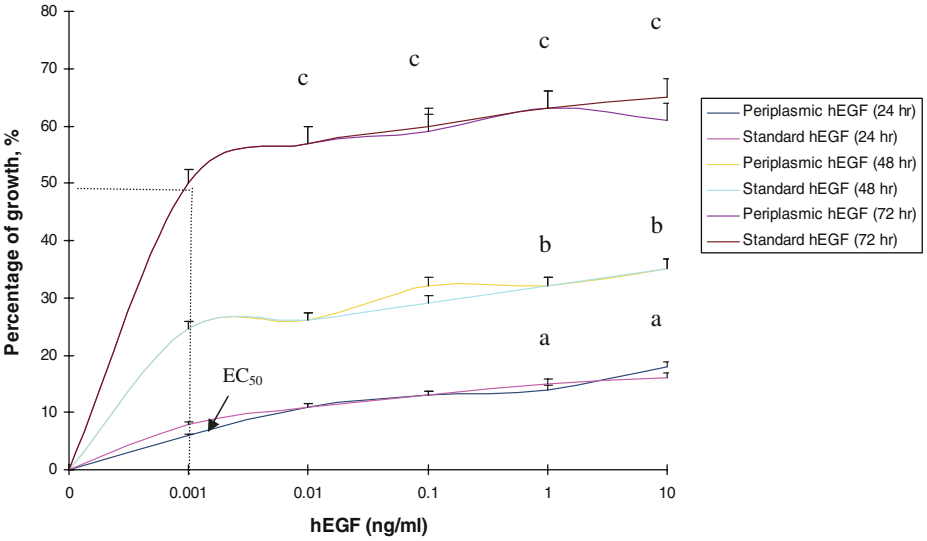

Fig. 6 Comparison percentage of growth and EC_{50} of HDF incubated with hEGF (commercial) and periplasmic hEGF (expressed) after 24, 48, and 72 h incubation. *a* Significantly different ($p<0.05$) at 10 and 1.0 ng/ml compared with 0.001 ng/ml hEGF concentration. *b* Significantly different ($p<0.05$) at 10 and 1.0 ng/ml compared with 0.01 and 0.001 ng/ml hEGF concentrations. *c* Significantly different ($p<0.05$) at 10, 1.0, 0.1, and 0.01 ng/ml compared with 0.001 ng/ml hEGF concentrations

Table 2 Percentage of growth of HDF incubated with hEGF (commercial) at 24, 48, and 72 h incubations.

Incubation (h)	hEGF (ng/ml)				
	C1 10	C2 1	C3 0.1	C4 0.01	C5 0.001
24	16%*	15%*	13%	11%	8%
48	25%*	32%*	26%	35%	29%
72	65%*	63%*	60%*	57%*	50%
$p < 0.05$	S	S	S	S	S

C Concentration, *S*, Significant

*Significantly different at $p < 0.05$ vs percentage at C5

Discussion

In the present study, recombinant hEGF was expressed into periplasmic space of *E. coli* using an amino-terminal expression vector. This vector contains OmpA secretion signal for the secretion of FLAG fusion proteins into periplasmic space of *E. coli*. Interferon alpha 2 and 6 were secreted across the cytoplasmic membrane into the periplasmic space of *E. coli* when the mature protein was fused to the signal peptide of OmpA, a major outer membrane protein of *E. coli* [10, 11]. Similarly, the expressed hEGF could be secreted into the medium quickly and efficiently, because of its lower molecular weight [12].

Over the years, fusion protein technology has become a tool for solving problems connected to recombinant protein production. A number of gene fusion systems have been developed to benefit from the molecular interactions arising from gene fusion technology. These systems include various extensions of the target protein, leading to an increase in solubility and yield of the protein to be expressed. Moreover, the properties of the additional tag facilitate identification and provide a one-step purification procedure of the protein by passing cell extracts or supernatants through columns of an appropriate matrix, e.g., affinity. Because of several factors such as the possibility of an adverse effect of the fusion on protein function, removal of the tag is required through consideration. On the other hand, the FLAG™ tag shows all the advantages and disadvantages of immunoaffinity purification. Although highly selective, the binding capacity was low, making scale up a costly undertaking. In addition to cost and low capacity, large-scale immunoaffinity chromatography applied to the production of therapeutic proteins has several disadvantages: ligand leakage, instability, and need for validation of antibody production. The stability of

Table 3 Percentage of growth of HDF incubated with periplasmic hEGF (expressed) at 24-, 48-, and 72-h incubations.

Incubation (h)	Periplasmic hEGF (ng/ml)				
	C1 10	C2 1	C3 0.1	C4 0.01	C5 0.001
24	18%*	14%*	13%	11%	6%
48	25%*	32%*	26%	35%	32%
72	61%*	63%*	59%*	57%*	50%
$p<0.05$	S	S	S	S	S

C Concentration, *S*, Significant

*Significantly different at $p < 0.05$ vs percentage at C5

the affinity chromatography column depends on the nature and source of the crude extracts. Furthermore, the FLAG™ tag, designed to be immunogenic, must be removed from the therapeutic proteins. In most cases, this can be accomplished with enterokinase. Despite these drawbacks, the FLAG fusion protein was useful in research and development, which can be readily purified and assayed by ELISA or any other immunochemical detection method, thus expediting the raising of antisera against a desired protein and characterization studies [13].

In this study, expression was conducted in small volume for preliminary study of production recombinant hEGF protein using pFLAG-ATS expression system in *E. coli*. This finding would make it possible to optimize and proceed to large-scale production for commercial purposes using the bioreactor system. This is because currently, there is no study performed using pFLAG-ATS expression system for commercial purposes, and the synthesized protein was proven to be bioactive. In general, a maximum hEGF yield of 250 mg/l was obtained by Yadwad et al. [14] in pH-controlled laboratory fermenters containing MBL medium.

A biological assay is a biological testing procedure for estimating the concentration of a pharmaceutical active substance in a formulated product or bulk material. In contrast to the common physical or chemical methods, detailed information on the biological activity of a substance is achieved. Over the last decade, biological assays have become more important to an effective quality control program in biopharmaceutical development and manufacturing. The general approach of most biological assays is to perform a dilution assay, which measures the biological responses at several doses [15]. A key assumption of a dilution assay is that the active component follows the same principle of activity in standard and sample preparation. In such cases, the unknown preparation may in theory be derived by diluting it with inert components or by concentrating the bulk solution. This concept may be checked with the help of a parallel-line model suitable for the analysis of the results obtained by several biological assays [16]. In the present study, MTT cell proliferation assay on HDF had been used to determine the biological activity of hEGF expressed in *E. coli*. Selection of growth factor concentrations for tenfolds dilution was extrapolated from in vitro and in vivo studies research protocols.

Study was conducted onto HDF cultures to determine the biological activity of hEGF synthesized in *E. coli* by using MTT cell proliferation assay. In this study, the incubation of HDF with different hEGF concentrations was carried out. This is to study the effect of different hEGF concentrations in stimulating cell proliferation. In the present study, MTT cell proliferation assay for 24 h incubation showed significant increase of cell proliferation, which occurred even at the highest concentration of 10 ng/ml. When the incubation period was increased to 48 h, the proliferative effect of hEGF on HDF at concentration 10 ng/ml was found to be significantly higher compared with concentration at 0 ng/ml with 1.35-fold, 1 and 0.1 ng/ml with 1.32-fold, 0.01 ng/ml with 1.26-fold, and 0.001 ng/ml with 1.2-fold. Similarly, when the incubation period was extended to 72 h, significant proliferative effect of hEGF was found at concentration 10 ng/ml compared with that at concentration 0 ng/ml with 1.61-fold, 1 ng/ml with 1.63-fold, 0.1 ng/ml with 1.59-fold, 0.01 ng/ml with 1.57-fold, and 0.001 ng/ml with 1.5-fold. These findings suggested that by increasing the concentration of hEGF, more HDF proliferation occurred as shown by the increase in absorbance values at 570 nm. Even at the highest concentration of hEGF (10 ng/ml), proliferation of HDF still occurred. These results were consistent with Yoon et al. [17]. They found that the amount of tyrosine-phosphorylated proteins was increased in the purified recombinant hEGF-treated cells compared with the commercial recombinant hEGF (10 ng/ml)-treated cells.

In addition, the results from this study showed that 10 ng/ml of hEGF concentration resulted in the 1.2-fold increase of cell proliferation at 24 h incubation, 1.35-fold increase at 48 h incubation, and further increase of 1.61-fold at 72 h incubation. This finding suggested that the bioactivity of hEGF in promoting cell proliferation of HDF cultures at 24, 48, and 72 h of incubations. These results were comparable with Ebrahimi-R. et al. [18]. They found the secreted EGF was bioactive in producing mitogenic effect on fibroblast cells after incubation conducted at 24, 48, and 72 h.

Another interesting finding was that the incubation at 72 h was found to be efficient period in producing the highest number of cell proliferation as well as the percentage of growth. In turn, the effective dose (EC_{50}) of hEGF in promoting growth on HDF was found at 0.001 ng/ml during this period of incubation. However, this finding was contradicted by Huang et al. [19]. They found that the hEGF exerts its biological activity in the concentration range 0.5–25.0 ng/ml. Even so, this finding is considered notable because it suggested that only a small amount of hEGF was needed to promote HDF growth effectively. In addition, the findings showed that of each hEGF concentration, the percentage of HDF growth was found to be significantly different between different duration of incubations.

Besides, the present study showed the rate of cell proliferation in nontreated cultures was significantly lower than the treated cultures with hEGF. This finding was in consistent with Sumi et al. [20]. They reported that the rate of cell proliferation was minimal in the absence of hEGF, suggesting virtually no response of cells to the growth-stimulating factors being present in the serum. They also demonstrated that hEGF synthesized in the bacterial cells exhibited the same physiological effects as mEGF with various biological activities, e.g., stimulation of cell proliferation and induction of DNA synthesis in human foreskin fibroblasts. These strongly showed that the hEGF had a complete and authentic structure. In conclusion, they suggested that the bacterially produced polypeptide could be applied to a broad range of investigations on the mechanism of diverse actions of EGF as well as its clinical treatments.

Furthermore, the concentration-response curves of mitogenesis for hEGF and five hEGF derivatives revealed an equivalent mitogenic activity compared with authentic hEGF [21]. Interestingly, the present study showed that the effect of hEGF on cultures of HDF was concentration dependent. Our finding demonstrated that the percentage of growth of HDF cultures was increased proportionately with the increase in the concentration of growth factor. This finding was consistent with Svoboda et al. [22] who found that the induction of [^{3}H] thymidine incorporated into the DNA of 3T3 cells was increased by increasing EGF protein concentration.

Another important point is that the results from this study showed that there was no significant difference for the percentage of growth in the comparison between commercial hEGF and periplasmic hEGF on HDF. Similar findings were conducted and reported by Engler et al. [23]. The present study showed that the hEGF secreted from *E. coli* was comparable in its biological activity on HDF to commercial hEGF produced in *E. coli*. This finding was in agreement with Ebrahimi-R et al. [18]. They found that the bioactivity of secreted hEGF was consistent with authentic protein that indicated the native conformation of the protein with the three disulfide bridges.

In this study, periplasmic hEGF was used instead of cytoplasmic hEGF to determine the biological activity of hEGF expressed in *E. coli*. This was because of previous studies, which suggested that the periplasmic hEGF was biologically active compared with cytoplasmic hEGF. Other studies are included in Ebrahimi-R et al. [18] who found that the bioactivity of soluble EGF in cytoplasmic expression was measured as only 17% similar

when compared with standard EGF biological activity. Competitive receptor-binding assay using ^{125}I-labeled mouse EGF showed that there was no activity observed in the mixture of cytoplasmic and membrane fractions of *E. coli* cells [24]. The periplasmic hEGF was comparable in its biological activity to human urogastrone and mEGF compared with cytoplasmic hEGF [25].

At present, this study promotes the use of pFLAG expression system, which is able to secrete the biologically active form of hEGF, with cysteines participating in proper folding by using periplasmic expression system. This system was also able to successfully translocate mature, correctly processed, and folded hEGF that induced proliferative responses in HDF cells. The disulfide bonds in hEGF generated the loops presenting important residues for hEGF-receptor binding, thus the proliferative effects of hEGF is the outcome of right folding and correct disulfide bridges.

In summary, this study suggested the advantages of using pFLAG-ATS expression system in the production of commercial recombinant hEGF protein, for the following reasons: (1) protein expressed is to be accumulated in the periplasmic space, (2) amino-terminal residues of the secreted peptide were identical to the natural peptide, (3) osmotic-shock procedure was a simple method for obtaining the desired protein from periplasmic space, (4) purification of the protein from the cold-water wash is easy, and recovery is high because the periplasmic proteins comprise only 4% of the total proteins of *E. coli* [26]. In addition, there are features and applications of pFLAG-ATS expression system that make it useful for the affinity purification and immunological detection of FLAG fusion proteins as described by Einhauer and Jungbauer [13]: (1) efficiency, (2) versatilities, (3) minimal effect on protein function, (4) ease of detection, (5) mild purification, (6) ease of removal, and (7) multiple applications, which are useful for further study of protein–protein, protein–DNA interactions, protein surveillance, and ultrastructure.

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