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Expression of Human Stem Cell Factor with Recombinant Baculovirus in BmN Cell Line and Silkworm

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A recombinant transfer vector pBacSCF was constructed by inserting huamn stem cell factor (hSCF) cDNA into plasmid pBacPAK8. BmN cells were co-transfected with modified *Bombyx mori* nuclear polyhedrosis virus (BmBacPAK) DNA and the recmbinant transfer vector to construct a recombinant baculovirus containing hSCF gene. DNA dot blotting and RNA dot blotting demonstrated that the hSCF gene was contained in the recombinant virus and transcribed. The recombinant baculovirus was infectious to BmN cells and to silkworm. SDS-PAGE analysis showed a specific band of expressed product in the extract of infected cells and in the heamolymph of infected larvae. Bioactivity of the recombinant hSCF was determined with TF-1 cell line and MTT colorimetric method in synergy with interlukin-3 (IL-3). These results revealed that the hSCF gene was over-expressed in cultured cells and lavae of silkworm.

Key words : Human stem cell factor, Baculovirus vector expression system, Expression

Introduction

Stem cell factor (SCF) is a multi-functional cytokine that acts in early period of hematopoiesis. It stimulates proliferation of hematopoietic stem cells, T lymphocytes, precursor B lymphocytes and melanocytes, stimulates proliferation and differentiation of mast cells, promotes maturation of macrophage and acts on formation of germ cells. Preclinical studies has demonstrated that SCF can cure anemia, recover marrow hematopoietic function after irradiation therapy and stimulate in vitro proliferation of hematopoietic stem cells. SCF works in synergy with many other cytokines. For example, it stimulates in combination with macrophage colony-stimulating factor (M-CSF), interlukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF) the proliferation of macrophages and granulocytes, stimulates in combinantion with thrombopoietin (TPO) the proliferation of megakaryocytes and stimulates in combination with erythropoietin (EPO) the proliferation of erythrocytes (Broudy *et al.*, 1997). Human SCF (hSCF) has been expressed in COS cells (Martin *et al.*, 1990; Zhu *et al.*, 1993), CHO cells (Arakawa *et al.*, 1992), *Escherichia coli* (Zhu *et al.*, 1993) and Sf9 cells (Zhang *et al.*, 2000).

We constructed a recombinant *Bombyx mori* nuclear polyhedrosis virus carrying hSCF gene and expressed recombinant hSCF (rhSCF) in BmN cells and in larvae of silkworm and determined the bioactivity of the expressed product with TF-1 cell line and MTT colorimetric method in synergy with IL-3.

Materials and Methods

Plasmid, virus, cell line and chemicals

Plasmid pUcSCF carrying human stem cell factor cDNA and transfer vector plasmid pBacPAK8 were propagated in *Escherichia coli* TG1. *Bombyx mori* N (BmN) cells were grown at 27°C in TC-100 medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL) (Summers and Smith, 1987). Modified wild-type *Bombyx mori* nuclear polyhedrosis virus, Bm-BacPAK,

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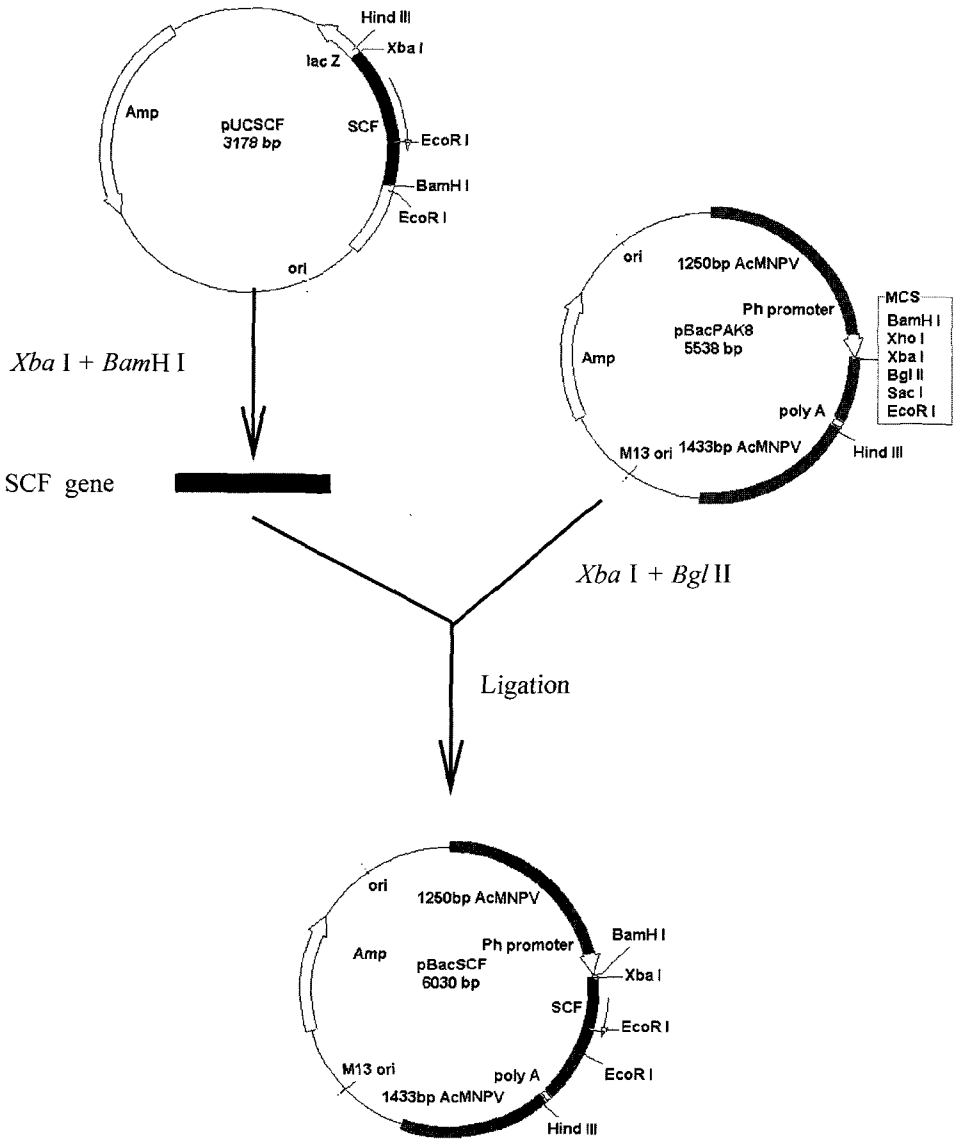

Fig. 1. Strategy for construction of recombinant transfer vector pBacSCF.

whose polyhedrin gene was replaced with a *Lac* Z gene, was propagated and titered in BmN cells. Restriction endonucleases, klenow enzyme, T4 DNA linkase, Dosper liposomal co-transfection reagent and DIG-High Prime DNA Labeling and Detection Starter Kit II were the products of Boehringer Mannheim company. hIL-3 was bought from PeproTech EC company.

Construction of recombinant transfer vector pBacSCF

The strategy for construction of recombinant transfer vector pBacSCF is shown in Fig. 1. Plasmid pUcSCF containing hSCF cDNA was digested with restriction endonuclease *Xba*I and *Bam*HI. A fragment of about 0.5 kb was recoverd and ligated into multi-cloning site of pBacPAK8 between the sites of *Xba*I and *Bgl*II. Then the recombinant plasmid DNA was transformed into competent cells of *E.coli* TG1. The recombinant transfer vector was identified by restricted digestion and agarose electrophoresis.

Extraction and linearization of BmBacPAK DNA

Propagation of BmBacPAK and extraction of its DNA were conducted following the procedure described by Summers and Smith (1987). The extracted BmBacPAK DNA was linearized by digestion with restriction endonuclease *Aoc*I at 37°C for 2.5 hrs. Then the digested sample was kept in water bath of 65°C for 10 minutes to inactivate the enzyme.

Construction of recombinant virus by co-transfection

The linearized BmBacPAK DNA was used to co-transfect

the BmN Cells with recombinant plasmid pBacSCF under the mediation of Dosper liposomal co-transfection reagent (Kitts *et al.*, 1993). Then the cells were incubated at 27°C for 3-4 days and checked for signs of infection.

Screening and identification of recombinant virus

When the infection was at an advanced stage, the recombinant viruses were screened by plaque assays (Kitts *et al.*, 1993). Then the recombinant viruses were identified by DNA dot Blotting with DIG-High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim) and by RNA dot blotting with ^{32}P-labeled probe (Sambrook *et al.*, 1989).

Expression of Recombinant hSCF (rhSCF) in cultured cells and in silkworm larvae

BmN cells were seeded into 25 cm^2 flask at a density of 3 $\times 10^6$ cells per flask and allowed to attach, then infected with recombinant virus at a multiplicity of infection (MOI) of 10 and cultured at 27°C. Three flasks of culture were taken out every day to collect culture medium and cells respectively for bioactivity determination of the expressed products. Culture medium was transferred to Eppendorf tubes and cells were kept in PBS (0.01 mol/L, pH7.4). Both the medium and the cell samples were stored at the temperature of -20°C.

Recombinant viruses propagated in BmN cells were mixed with ampicillin to a final concentration of 100 μg/ml and used to infect fifth instar larvae of silkworms. The larvae were fed at 24-25°C on mulberry leaves sprayed with 500 u/ml of chloromycetin for 24 hrs then on normal leaves. Ten larvae both male and female 5 each were sampled to collect their haemolymph. The haemolymph was mixed with a small drop of β-mercaptoethanol and stored at the temperature of -20°C for determination of bioactivity.

Analysis of rhSCF by SDS-PAGE

The samples of the infected cell culture or haemolymph of infected larvae was mixed with Laemmli buffer and boiled for 5 minutes and centrifuged at 10,000 rpm for 10 minutes, then analyzed for the expressed rhSCF by SDS-PAGE on 15% polyacrylamide gel (Laemmli, 1970).

Pre-treatment of samples

The supernatant of cell culture was used to determine rhSCF activity directly after centrifugation. The cell samples were treated twice, 30 seconds each, with ultrasonic in ice bath and centrifuged at 4°C, 10,000 rpm for 10 minutes. The supernatant was transferred to a sterilized Eppendorf tube for determination of activity of expressed rhSCF.

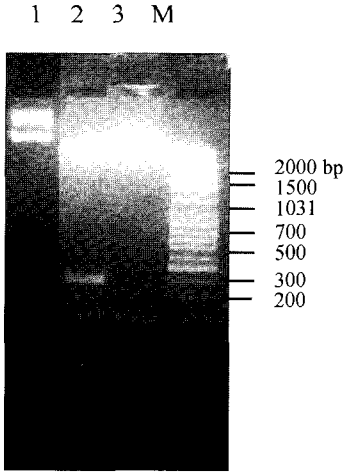

Fig. 2. Electrophoresis profiles of recombinant transfer vector pBacSCF. 1 pBacSCF; 2. pBacSCF/*Xba*I+*Eco*RI; 3 pBacSCF/*Eco*RI. M. Marker.

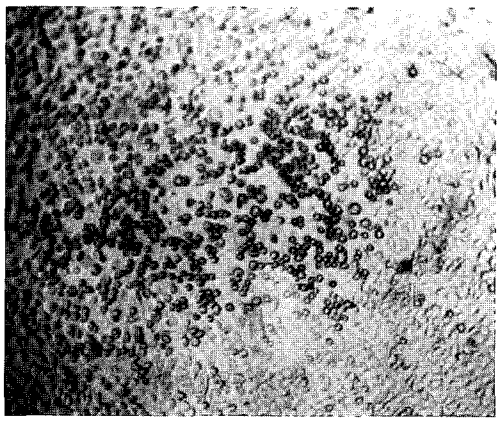

Fig. 3. Plaque formed by recombinant virus.

The haemolymph of silkworm larvae was centrifuged at 4°C, 12,000 rpm for 10 min. The supernatant was transferred to a new tube and diluted 10-fold with PBS(0.01 mol/L), pH 7.4and centrifuged again. Then the supernatant was stored in sterilized Eppendorf tube and used to determine the activity of expressed rhSCF.

Detrmination of bioactivity

Determination of bioactivity of rhSCF was conducted following the procedures as decribed by Zhang Dongmei *et al.* (2000). TF-1 cells grown in RPMI1640 medium with 10% FBS and 80 U/ml rhGM-CSF were washed three times with serum-free RPMI1640 medium, then diluted to a density of 4×10^5 cells/ml with RPMI1640 containing 20% FBS and 0.25 ng/ml IL-3. The prepared expression samples were 2-fold diluted in series on 96 well plate with RPMI1640 medium and 50 μl was left in each hole. 50 μl of diluted cells was added into each hole, mixed and cul-

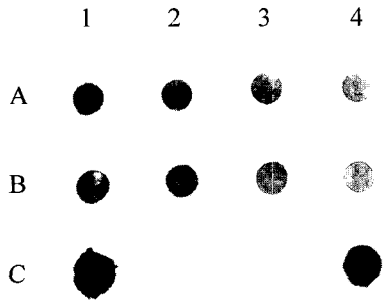

Fig. 4. Analysis of recombinant virus by do blotting. Al~A4, B1~B4: Total DNA from cells infected with recombinant virus; C1: pBacSCF; C2 Total DNA of mock-infected cells; C3: Total DNA from cells infected with wild-type virus; C4 pUcSCF.

tured at 37°C, 5% CO_2 for 48 hrs. 10 μl MTT solution (5 mg/ml) was added to every hole and the cells were cultured for 5 hrs more. Then 100 μl 10% SDS solution (10% SDS, 0.01 mol/L HCl) was added to each hole. The plate was kept at 37°C, 5% CO_2 overnight and O.D value at 570 nm was measured. A dose-effect curve was made and the activity of rhSCF was calculated.

Results

Construction of recombinant transfer vector pBacSCF

The structure of the recombinant transfer vector pBacSCF was shown in Fig. 1. The start coden ATG of the SCF gene follows downstream the promoter of the polyhedrin gene. Restriction endonuclease *Bam*HI and *Bgl*II are isoschizomers and the cohensive ends produced by them can bind to each other. Once the cohensive ends were ligated, the resulting site could not be cut by either of the two enzymes any more. Therefore, the *Eco*RI site downstream *Bgl*II on pBacPAK8 was used to identify the recombination. There is another *Eco*RI site inside the hSCF cDNA fragment and is 170 bp apart to the one on pBacPAK8 itself. Thus, the recombinant plasmid was cut by restriction endonuclease *Xba*I and *Eco*RI, two fragments sizing 375 bp and 170 bp respectively were resulted as indicated in Fig. 2. These revealed that hSCF cDNA fragment was inserted into the transfer vector pBacPAK8 in a correct direction. The resulting recombinant transfer vector was named pBacSCF.

Co-transfection and screening of recombinant virus

BmN cells were co-transfected with the recombinant transfer vector pBacSCF and linearized BmBacPAK DNA under mediation of Dosper transfection reagent, then cultured at 27°C. The symptoms of infection appeared about 4 days after co-transfecton.

The supernatant of the culture was used to screen

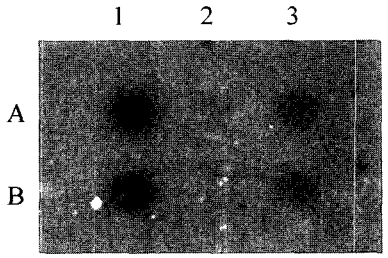

Fig. 5. Analysis of hSCF mRNA by dot blotting. Al: pBacSCF; B1: pUcSCF; A2: Total RNA of mock-infected cells; B2: Total RNA from the cells infected with wild-type virus; A3, B3: Total RNA from the cells infected with recombinant virus.

recombinant virus by plaque assay. The plaque formed by the recombinant virus is as shown in Fig. 3. Total 30 white color plaques were picked out and the recombination rate was 100%. The plaques formed by wild-type virus were blue colored by X-gal staining. The plaque assay was conducted two rounds and all the plaques formed by recombinant virus were white colored.

Analysis of recombinant virus by dot blotting

BmN cells were infected with recombinant virus at a MOI of 5. Their total DNA was isolated and analyzed by dot blotting with the probe labeled with DIG-reagent. All the 8 samples tested showed positive reaction as shown in Fig. 4, while total DNA isolated from mock-infected cells and cells infected with wild-type virus all showed negative reaction. These indicated that the hSCF gene was inserted into the genome of the recombinant virus.

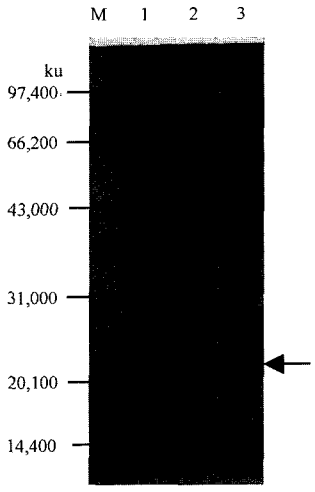

Fig. 6. Analysis of rhSCF expressed in BmN cells by SDS-PAGE M: Marker; 1. Extractof cells infected with wild-type virus; 2. Extract of cells infected with recombinant virus (arrow indicates the band of expressed rhSCF); 3. Extract of cells mock-infected.

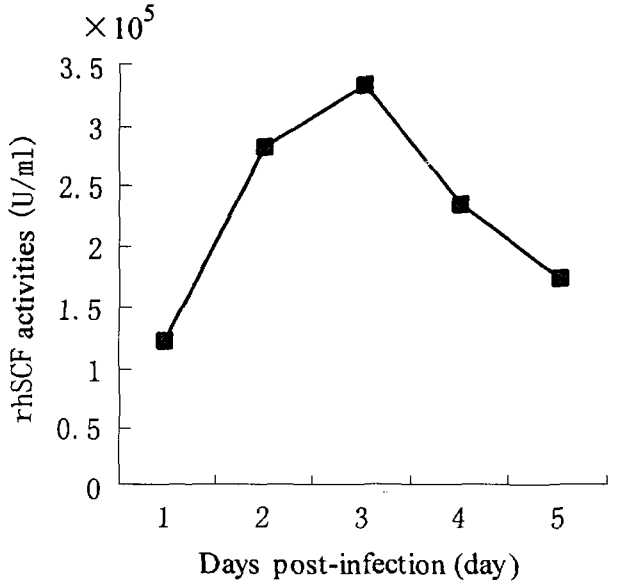

Fig. 7. Time curves of expression of rhSCF in silkworm larvae.

Infectivity of the recombinant virus to BmN cells and to silkworm larvae

The recombinant virus constructed in this study was highly infectious to both BmN cells and to silkworm larvae. To cultured cells, the symptoms appeared from the third day post-infection and the cells detached from wall of culture flask and floated in culture medium. To the fifth instar larvae, the symptoms appeared in the third to fourth day post-infection. In the fourth to fifth day, the infected larvae crawled everywhere quickly and their body wall was easily broken with milky haemolymph running out. It is, therefore, suitable to collect haemolymph before the body wall is broken when the silkworm larvae are used to express rhSCF.

Analysis of hSCF mRNA by dot blotting

Total RNAs were isolated respectively from cells infected with recombinant virus, cells infected with wild-type virus and mock-infected cells with Trizol reagent and identified by dot blotting hybridization with ^{32}P-labelled probe. As shown in Fig. 5, the hSCF cDNA containing plasmids pUc-SCF and pBacSCF were all positive and total RNAs from wild-type virus infected cells and mock infected cells were negative, while the total RNAs isolated from the cells infected with recombinant virus were hybridized with the probe and showed positive reaction. These results revealed that hSCF gene was not only recombined into the genome of the baculovirus, but also transcribed along with the multiplication of the recombinant virus.

Analysis of expression product by SDS-PAGE

Recombinant viruses were inoculated to BmN cells at a MOI of 10 and the cells were collected after 72 hrs of infection. Haemolymph of silkworm larvae infected with the recombinant virus was collected after 72 hrs of inoculation. Both the cell and haemolymph samples were subjected to SDS-PAGE analysis. Two bands with the molecular weight about 22 kDa and 24 kDa appeared to the cell samples on the SDS-PAGE profiles and only one band of 24 kDa to the haemolymph samples as shown in Fig. 6.

Determination of bioactivities of expression products

The culture supernatant and cell extract of recombinant virus infected cells and the haemolymph of recombinant virus infected silkworm larvae all showed hSCF activity as determined with TF-1 cell line and MTT colorimetric method. The expression products supported proliferation of TF-1 cells. The samples of wild-type virus infected cells and silkworm larvae did not support the growth and proliferation of TF-1cells. These results indicated that the recombinant virus expressed rhSCF in cell culture and in silkworm larvae.

rhSCF amount corresponding to half maximal optical densities was designated one unit per ml. The expression level of hSCF gene in silkworm larvae increased gradually and reached the highest at about 3.3×310^5 U/ml in the third day after infection with the recombinant virus, then decreased afterward as shown in Fig. 7.

Discussion

As described above, a recombinant virus carrying hSCF gene was successfully constructed by co-transfect recombinant transfer vector and linearized BmBacPAK DNA into BmN cells. The recombinant virus was screened by plaque assay and identified by DNA and RNA dot blotting hybridization. The recombinant virus was highly infectious to BmN cells and to silkworm larvae. Both the culture supernatant and cell extract of recombinant virus infected cells and the haemolymph of recombinant virus infected silkworm larvae have the bioactivities of hSCF. These results revealed that the hSCF gene contained in the recombinant virus was correctly transcribed and expressed along with the multiplication of the recombinant viruss.

The expression products from BmN cells showed two bands of 22 kDa and 24 kDa on SDS-PAGE profiles, while, the expression products from haemolymph of infected larvae have only one band of 24 kDa. It was speculated to be resulted from the glycosylation of different extent in cultured cells and in silkworm larvae. Studies have revealed that SCF is a glycosylated protein with two glycosylation sites in the molecule (Arakawa *et al.*, 1992). rhSCF expressed in BmN cells was glycosylated in a

lower level and the expression products were the mixture of molecules of different glycosylation. While, rhSCF expressed in silkworm larvae was glycosylated highly and all the molecules were glycosylated and present as one band of about 24 kDa on SDS-PAGE profiles.

Because SCF has important biological and clinical effects, scientists in many research institutions have tried to establish effective ways to express it in large scale. The baculovirus expression vector system which was developed in 1980s is characterized with high expression efficiency and perfect post-translational modification of the expressed products (Smith *et al.*, 1983). It has been widely used to express useful genes in insect cell culture and in insect larvae. *B. mori* nuclear plyhedrosis virus belongs to the baculovirus family and silkworm larvae are bigger in size than other insects and can be reared easily. It is therefore more advantageous to use the *B. mori* baculovirus expression vector system to express foreign genes from many sources. hSCF gene was successfully expressed in higher level in both silkworm cell culture and larvae in this study. It is highly hopeful to establish a expression system for large scale production of rhSCF with the recombinant *B. mori* nuclear polyhedrosis virus and the silkworm.

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