A three-step purification method of large quantities of human recombinant α endothelial cellular growth factor for clinical use

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Abstract. The endothelial cellular growth factor α-ECGF is a candidate drug for the induction of therapeutic neoangiogenesis. Its use in extensive experimental and clinical trials is hampered by the fact that currently published purification procedures allow only small yields, and the absence of pyrogenic impurities is not demonstrated. The rh α-ECGF was expressed in *E. coli*. Isolation of rh α-ECGF from *E. coli* lysates to apparent homogenicity was achieved by a three step purification procedure involving ionic exchange, heparin-sepharose and polymyxin B chromatography. By this method, 200 mg of rh α-ECGF was purified from 15 g wet weight *E. coli* bacteria. The isolated protein of 18 kDa appeared as a single band after SDS gel electrophoresis and subsequent silver-staining. The biological activity was expressed in the chorion-allantois-membrane assay and in the ^{3}H-thymidine proliferation in baby hamster kidney cells. Drug trials with rabbits revealed no increase in body temperature after intravenous injections with 1 mg rh-ECGF.

Introduction

Compromised microvascular circulation is an important component of several cardiovascular diseases including syndromes of acute coronary ischemia (1), hypertensive heart disease with left ventricular hypertrophy (2), chronic congestive cardiac failure (3), and diabetic peripheral vascular insufficiency (4).

In ischemic regions small arterioles and capillaries begin to grow. Polypeptide growth factors are thought to play an important role in the collateralization processes in response to ischemia. The heparin-binding growth factor family consists of 10 identified mitogens (5-8) and 4 homologs with unknown functions (9). Of these growth factors, acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2), and vascular endothelial growth factor (VEGF) constitute the most widely studied group. The heparin-binding growth factors α-ECGF and ß-ECGF were first separated from bovine brain tissue by high-pressure liquid chromatography (10). α-ECGF and FGF-1 are processed out of α-ECGF by posttranslational processing (10). The strong relationship between these three factors is shown by the cross-reaction of FGF-1 with ECGF-antibodies (10). α-*ECGF*, ß-ECGF, FGF-1 and FGF-2 are potentiated by the glycosaminoglycan heparin. α-ECGF and FGF-1 are polypeptides of a similar molecular weight, 17 kDa and 20 kDa respectively. The heparin-binding growth factors appear to be cellular peptides with a mitogenic potency to mesoderm- and neuroectoderm-derived cells (11). FGF-1 and FGF-2 can induce the growth of endothelial, smooth muscle cells and fibroblasts and show a chemotactic effect on these vascular cells (12-14). Endothelial cells possess a high-affinity for these mitogenic peptides through specific receptors for FGF-2 (FGFR) on their vascular surface (15). Polysaccharide-heparansulphates appear to initiate the following signal transduction by binding FGF/FGFR and ligand-induced dimerisation of the receptor (16). The connection between heparansulphate and the extracellular FGFR-kinase induces the transphosphorylation of FGFR, which is responsible for the specificity and ligation of FGF (17).

Studies concerning the induction of new vessel formation by application of FGF-1, FGF-2 and VEGF in the ischemic heart have been promising. Intracoronary FGF-2 injections in ischemic dog myocardium reduced the size of the infarct and increased the number of capillaries (18). Lopez and colleagues achieved a functional improvement of left-ventricular parameters in ischemic porcine myocardium by FGF-1 (19). Under coronary angiography, Schumacher and associates, showed a significant growth of new vessels by FGF in the rat heart (20). In clinical studies the intramyocardial injection of FGF-1 induced the growth of new capillary networks in patients suffering from coronary heart disease (21). The application of FGF-2 reduced the size of the ischemic area and the incidence and/or severity of angina pectoris attacks (22).

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The purification of α-ECGF from bovine brain was first described by Gospodarowicz (23). High-pressure liquid chromatography (HPLC) played the major role in the described method. In order to obtain greater amounts of ECGF for structural studies the HPLC was supplemented by heparin-sepharose affinity chromatography (10). The existing procedures were sufficient to purify relatively small quantities of mitogens (24-26). Before initiating the heparin-sepharose affinity chromatography, ECGF was pre-purified by acid-treatment, gel exclusion chromatography and ammonium sulfate precipitation (27). Previous methods of purification of ECGF by the aforementioned authors produced a single-chain polypeptide which possessed an anionic isoelectric point and a molecular weight of 20 kDa (24,25,28). The required quantities of ECGF for clinical and experimental studies were purified by combining HPLC and heparin-sepharose chromatography and other starting preparations (10,21). We now describe a fast purification method using ionic exchange, heparin-sepharose affinity and polymyxin B chromatography. In order to produce sufficient amounts of angiogenic growth factors for further clinical and experimental studies, the described method attempts to replace HPLC by ionic exchange and heparin-sepharose affinity chromatography. Extensive studies in this area require larger quantities of biologically active angiogenic mitogens that can be produced quickly and to a high level of purity. Removing HPLC from the suggested methodology also eliminates the possibility of *in vivo* angiogenesis, which is thought to be caused by the TFA buffer used in HPLC (29).

Materials and methods

Plasmid and bacteria. The plasmid for the expression of human α-ECGF in *E. coli* was obtained from Tom Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, MD). The α-ECGF was integrated in a pET-3c-vector from Stratagene Cloning Systems, La Jolla, USA.

The plasmid was transformed into *Epicurian Coli* BL 21(DE3)pLYSS-*E. coli* bacteria by electroporation (30). Transformed bacteria were grown on agar plates overnight and used the same day.

Induction of recombinant protein synthesis and preparation of induced cell extracts. One individual colony was expanded in each of 10 tubes in 50 ml LB medium (10g/l NaCl, 10g/l peptone, 5g/l yeast, 1% glucose, 100 μg/ml ampicillin, pH 7.5) for 6 h at 37˚C. From each tube a 5-ml sample was taken and analyzed for ECGF expression. The samples were grown to an optical density of 0.4 (A_{547nm}), 5 ml 1 M IPTG was added and the samples were shaken at 37˚C for 1.5 h. A 1-ml probe was taken from each tube, and was analyzed after centrifugation. It was then re-suspended in 100 μl lysis buffer by SDS electrophoresis for α-ECGF expression.

Two tubes with high ECGF expression were pooled and distributed into 20 1-l flasks containing 250 ml LB medium. The cells were expanded to an optical density of 0.4 (A_{547nm}) at 37˚C and 140 rpm. ECGF was induced by adding 200 ml 1 M IPTG (1 mM final concentration). After 3-h of incubation, the cultures were pooled and centrifuged (6000 x g, 10 min). The cell pellet (15 g) was suspended in 93 ml of disruption buffer (TE buffer, 1 mM EDTA, 13 mM Tris-HCl, pH 8.0) plus 36 g/l glucose, 10 mg/l lysozyme, 1 mM PMSA and shaken for 30 min at 4˚C. The cells were disrupted by a single passage through a french press at 15000 kp/cm^2.

Purification of recombinant α endothelial cellular growth factor from E. coli. After centrifugation at 45000 x g for 20 min, the supernatant (35 ml) was filtered through a 0.22-μm filter. The filtration was first followed by an ammonium sulfate precipitation (40% final concentration) (27). After centrifugation at 20000 x g for 20 min the supernatant was saturated with ammonium sulfate to 85% and centrifuged at 20000 x g for 20 min. The pellet was dissolved in 30 ml of distilled water. The solution was desalted over a sephadex G 25 column (2.7x30 cm) equilibrated with 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0. A cation exchanger (CM sepharose, C 50) column (2.6x10 cm) was equilibrated with 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0 at a flow rate of 3.5 ml/min. The desalted solution from the sephadex G 25 column was pumped on the column at a flow rate of 3.5 ml/min and washed with 200 ml equilibration buffer. Elution was performed on a linear gradient (3.5 ml/min, 30 min) mixed from 50 mM phosphate, 0.15 M NaCl, pH 7.0 and 50 mM phosphate, 0.5 M NaCl, pH 7.0. The peak fraction (90 ml) was collected and analyzed for α-ECGF content by ELISA.

The peak from cation exchanger chromatography was desalted by using a sephadex G 25 column. The elution fraction containing the ECGF was passed over a G 25 (HiPrep, 2.6x10 cm) column under the same conditions as described above.

The heparin-sepharose affinity chromatography column (2.6x20 cm) was packed approximately half with heparin-sepharose (Pharmacia, CL-6B) and equilibrated with 10 mM Tris-HCl, pH 7.0. The elution fraction from the G 25 column was pumped on the column with a flow rate of 3 ml/min and washed with 10 mM Tris-HCl, 0.15 M NaCl, pH 7.0 buffer until the adsorption at A_{280nm} reached baseline level. ECGF was eluted with 10 mM Tris-HCl, pH 7.0 buffer containing 2 M NaCl at a flow rate of 3 ml/min. The elution peak (200 ml) was collected and sterilized by passing through a 0.22-μm filter.

Removal of endotoxin impurities. An endotoxin-free column (BioRad, 0.5x10 cm) was packed with 1 ml polymyxine B suspended in pyrogen-free water (aqua pro injectable). The sterile filtered elution fraction from the heparin-sepharose column was pumped over the column with a flow rate of 1 ml/min and collected in sterile pyrogen-free tubes.

SDS-polyacrylamide gel electrophoresis and Western blotting. The polypeptides were separated in sodium dodecyl-sulfate polyacrylamide gels by a previously decribed method (31). The proteins were electroblotted onto a nitrocellulose membrane at 0.8 mA cm^{-2} in accordance to standard procedures. The detection of the proteins was performed by utilizing a monoclonal anti-aFGF-IgG antibody (Sigma, mouse, anti-human-FGF) at a dilution of 1:5000 and the anti-mouse-anti-IgG antibody (Dako, goat) at a dilution of 1:75. The membrane was blocked with 10% milk powder in 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20 (TBST) for 1 h at room temperature (RT). After washing, the membrane was incubated

with anti-human-FGF antibody diluted in 5% milk powder in TBST for 1 h at RT. The membrane was washed three times, and the second anti-mouse-anti-IgG antibody in a 1:75 dilution in 5% milk powder in TBST was added. After the 1 h incubation the membrane was washed and developed with a carbazol solution (basic carbazol solution, 0.1 M sodium acetate, 30% H_2O_2).

Alternatively the gels underwent silver-staining instead of Western blotting. A commercial kit from Pharmacia was used for the silver-staining and a silver marker kit from BioRad Lab was used according to the protocol.

ELISA. α-ECGF concentrations were determined by ELISA by plates coated with 200 µl/ml of rabbit-anti-aFGF. The plates were incubated overnight at 4˚C. The incubation was followed by a wash, carried out 3 times, using 2 M Tris-HCl, 10% Tween-20, 2% merthiolat, pH 8.0. The wells were blocked with 50 µl/well blocking buffer (10.5 g boric acid, 7 g NaCl, 500 µl Tween-20, 0.372 g EDTA, 2.5 g gelatine, 2% NaN_3/l distilled water) for 1 h at 37˚C. After the washing 50 µl/well of the positive controls (commercial aFGF) or 50 µl of the eluate was added and incubated for 1 h at 37˚C. After washing 50 µl/well of the second peroxidase-linked antibody (goat-anti-rabbit-anti-IgG, Conco) was added. After 1 h of incubation and washing, 50 µl/well substrate (OPD, substrate buffer, 30% 4.5 mM H_2O_2) was added and the plates were incubated for 20 min in the dark. Finally the reaction was stopped with 50 µl/well 4.5 mM H_2SO_4 and the α-ECGF concentrations were measured in the ELISA-reader at A_{492nm}.

Chorioallantoic membrane assay. The CAM assay was used to show the angiogenic potency of the purified α-ECGF. By using the established method of Auerbach, *et al* (32) and Wilting, *et al* (33) the effect of the growth factors was observed with a light microscope. Fertilized hen eggs were incubated for 13 days at 37.5˚C, 66.6% humidity. The eggs were prepared by cutting a window in the oval side of the shell after three days of incubation. The ECGF and heat-denatured ECGF (60˚C for 30 min) were dissolved in PBS buffer and applied on thermanox coverslips with a diameter of 5 mm. After air drying, the slips were turned upside-down on chorioallantoic membranes. The eggs were incubated for an additional three days and the membranes were removed from the eggs and fixed in fixation buffer (3% glutaraldehyde, 3% formaldehyde in 0.12 M sodium cacodylate buffer). The membranes were examined under a light microscope, magnification x10. The photographed angiogenesis was evaluated under blind study conditions using a score between 0 and 6.

^{3}H-thymidin-proliferation assay. Following an established technique (34) 200 µl/well of BHK-21 (baby hamster kidney) cells were seeded into 96-well plates at $1.5x10^4$/ml in growth medium (MEM with Glutamax-I, 10% fetal calf serum (FCS): 100 IU penicillin, 0.075% sodium bicarbonate, 1 mM sodium pyruvate, and 10 mM HEPES). After 24 h the growth medium was replaced by 200 µl starving medium (without FCS) and incubated for another 24 h. The starving medium was replaced by stimulating medium (1% FCS), and 20 µl/well α-ECGF was added. After 24 h of incubation the cells were marked by adding 1 Ci/well of methyl-^{3}H-thymidine and again incubated for 4 h. The medium was then removed by filtration with a FilterMate™ cell harvester. Each well was incubated with 100 µl 0.05% trypsine/0.02% EDTA at RT, and after 10 min the cells were harvested with the FilterMate cell harvester on a UniFilter™ plate. The filter was washed, dried, and 30 µl/well scintillation fluid MICROSCINT™ 20 from Packard was added and counted in a TopCount NXT™ .

Pyrogenicity testing of α-ECGF. The amount (1.1 mg) of 1 ml ECGF was injected intravenously into the ear veins of two New Zealand white rabbits. One additional rabbit was injected with sterile pyrogen-free PBS. Rectal temperatures were taken every 30 min for 3 h.

Results

Transformation. Earlier experiments have shown that only freshly transformed bacteria produced an optimal ECGF expression, therefore cultures were started immediately after transformation. However, as shown in Fig. 1 each of the 10 different clones collected after transformation produced significant amounts of ECGF. The α-ECGF expression and the amount of contaminating *E. coli* impurities varied to a large extent.

Clone Nr. 5 and 6 were selected for large-scale production. From 5 l of IPTG-induced *E. coli* suspension, 15 g of bacteria (wet weight) was obtained (Fig. 1).

Ionic exchange chromatography. By using a linear gradient of the sodium solution (from 0.15 M NaCl to 0.5 M NaCl) the peaks during cation exchange column chromatography were collected in an elution period. A first small peak was collected 9 min after starting the elution of the proteins. The α-ECGF fraction was eluted in a peak, which was detected 15 min after the start of the elution. The extinctions of the various samples, being collected during ionic exchange, were measured with a photometer (A_{280nm}). A photometric-defined protein concentration of 0.2 mg/ml elution was reached (Fig. 2).

Heparin-sepharose affinity chromatography. The protein peak of α-ECGF realized in exchange chromatography was achieved after desalting on a heparin-sepharose affinity chromatography column which further reduced the contamination with the endotoxin. Increasing salt concentrations (0.15 M, 0.6 M, and 1 M) were used to discern the optimal desalting concentration through comparison of protein peaks. The largest protein peak correlating to 200 mg of α-ECGF, was eluted using a 2 M NaCl concentration, 38 min after the start of elution (Figs. 3 and 4).

Proof of angiogenic and mitogenic potency. Three days after ECGF-application the membranes of the chicken embryos were examined under light microscopy. The membranes supplied with the growth factor showed a great variation in the vascular structure compared to the heat-denatured samples. α-ECGF induced an impressive growth of angiogenic structures which emanated radially from the membrane. A large number of neo-angiogenic vessels spread out of the

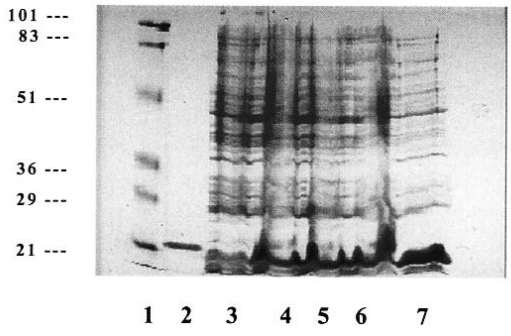

Figure 1. Samples of transformed BL21(DE)pLys-*E. coli* cells before and after IPTG-boostering. The proteins were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie Blue. The lanes show a distinct increase in α-ECGF (MG=17000 kDa) concentration after boostering. The human growth factor aFGF (MG=18000 kDa) served as a marker protein. Lane 1, molecular weight marker; lane 2, aFGF (18000 kDa); lane 3, *E. coli*-lysate before IPTG induction; lanes 4-6, lysate 1, 2 and 3 h after boostering; lane 7, product of high-pressure homogenization. The molecular weights of the size standard proteins are indicated.

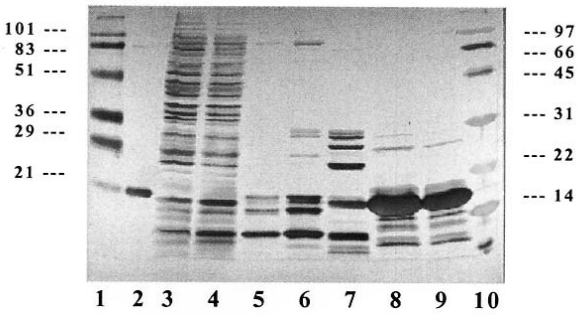

Figure 2. Samples on a silver-stained gel indicating the α-ECGF (MG=17000 kDa) concentration after ammonium sulphate precipitation and ionic exchange chromatography during the purification process. This shows an infinitely small loss of α-ECGF (17000 kDa) while pumping the lysate on the C-50 cation exchange column. The washing process with a 0.15 M NaCl salt solution caused significant elution of growth factor. A first pre-peak sample was collected during a linear salt gradient elution and was found to contain a distinct amount of α-ECGF quantity. Chromatography with a linear gradient of 0.15 M and 0.5 M NaCl concentrated the growth factor sample and withheld the majority of contaminating *E. coli* proteins from the sample. Lane 1, molecular weight marker; lane 2, aFGF (18000 kDa); lane 3, lysate before IPTG induction; lane 4, lysate after ammonium sulphate precipitation; lane 5, loss of proteins during packing of the column; lane 6, eluate during washing process with 0.15 M NaCl; lane 7, pre-peak during elution; lanes 8 and 9, main peak in gradient elution (α-ECGF=17000 kDa); and lane 10, silver marker proteins. The molecular weights of the marker proteins are indicated.

original host vessels. These changes were completely absent in the control group embryos, in which a normal developed reticular vascular web was discerned.

The ^{3}H-thymidine assay showed a dose-dependent 7-fold increase of DNA synthesis in the ECGF-stimulated cultures, whereas the assays without growth factor showed cessation of cellular proliferation. After heat denaturation of ECGF (60˚C, 30 min) a remaining proliferative activity was observed (Figs. 5-7).

Discussion

Ischemia is characterized by inadequate blood flow and tissue oxygenation that is typically the consequence of decreased

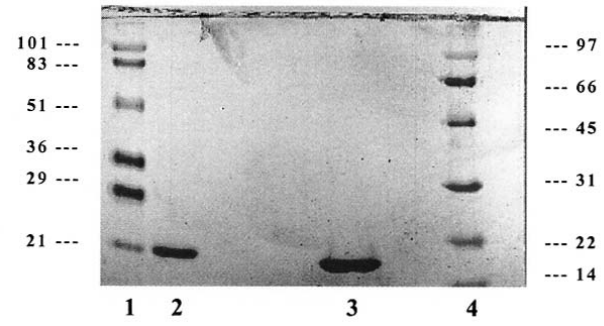

Figure 3. Silver-staining of human recombinant α-ECGF (17000 kDa) being eluted in heparin-sepharose affinity chromatography. The gel shows a growth factor lane of high purity. Lane 1, molecular weight marker; lane 2, aFGF (18000 kDa) standard; lane 3, α-ECGF peak of heparin-sepharose column; and lane 4, silver marker proteins.

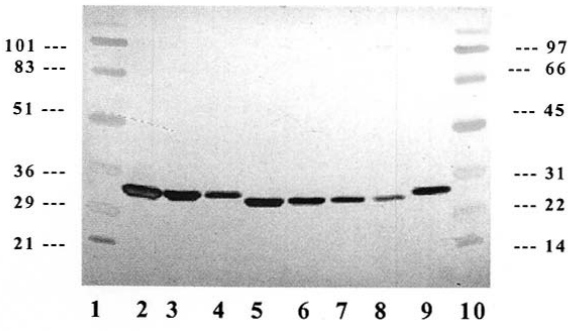

Figure 4. Silver-staining of human recombinant α-ECGF after endotoxin absorption in polymyxine B column. Lane 1, molecular weight marker; lanes 2-4 in polymyxine B column. Lane 1, molecular weight marker; lanes 2-4, aFGF standard [lane 2, aFGF (15 μg); lane 3, aFGF (7 μg); lane 4, aFGF (3 μg)]; lane 5-8, α-ECGF after polymyxine B gel filtration [lane 5, ECGF (15 μl); lane 6, ECGF (7 μl); lane 7, ECGF (3 μl); lane 8, ECGF (1.5 μl)]; lane 9, aFGF (3 μg); and lane 10, silver marker proteins.

patency of atherosclerotic vessels. The clinical strategies under present use work to increase blood flow in ischemic peripheral and cardiac muscle, and are largely dependent on vasodilation, angioplasty and surgical revascularization. Use of these successful approaches is in many ways restricted. Improvement in blood flow and muscle function could also be achieved by neovascularization, or angiogenic processes providing new collateral vessels. The therapeutic use of angiogenic growth factors has been the subject of intensive investigation over the last few years and has demonstrated promise as a potential modality to treat both acute and chronic myocardial ischemia. A number of studies have shown that by administering bFGF, collateral vessel flow and myocardial function have improved greatly in chronic myocardial and peripheral limb ischemia (19,35). The administration of vascular endothelial growth factor (VEGF) (36), aFGF (FGF-1) (20,21,37) and ECGF (29,38) has also been reported to produce functionally significant angiogenesis.

Our aim was to produce α-ECGF in a low-cost, fast and effective manner for clinical study towards a new treatment of coronary heart disease with growth factor application during elective bypass surgery. Fresh transformation in *E. coli*-BL21(DE3)pLYSS-cells without storage and selection of clones achieved a higher productivity of the cultures. High-pressure homogenization (French press) proved to be a more

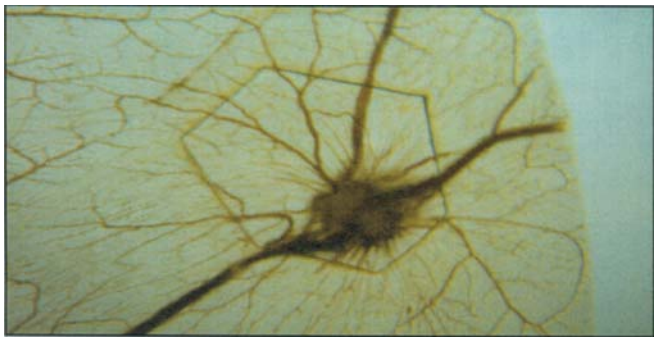

Figure 5. Chorionallantoic membrane assay with application of 6 μg α-ECGF.

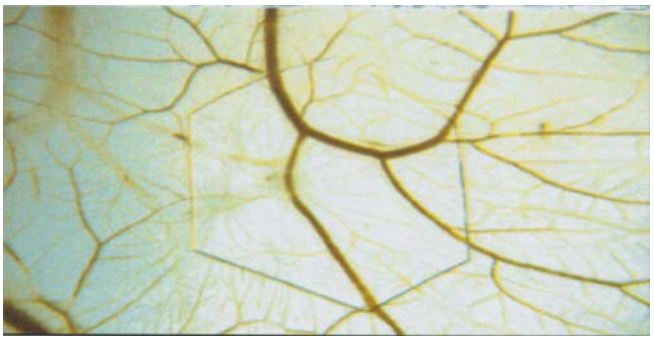

Figure 6. Chorionallantoic membrane assay with application of 6 μg heat-denatured growth factor.

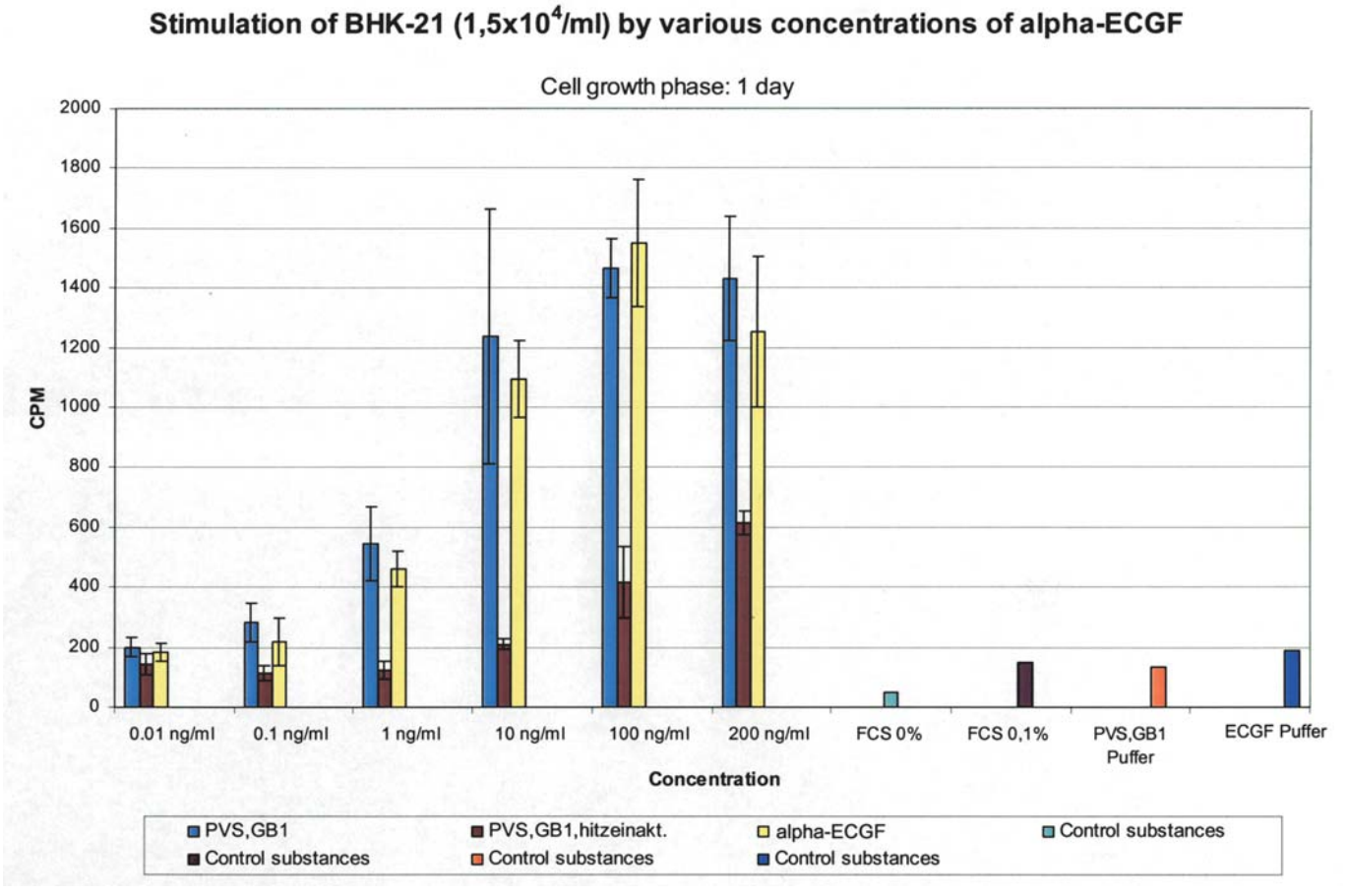

Figure 7. Rate of DNA synthesis in baby hamster kidney cells (1.5x10^4/ml) during a one-day cell growth and application of various concentrations of purified human recombinant α-ECGF.

efficient and effective method to release rh-ECGF in comparison to methods such as ultrasound sonification (39). The ammonium sulphate precipitation of the French press lysate eliminated some of the nonspecific bacterial proteins and concentrated the ECGF (34). Gel filtration in a G 25-med column removed the salt fraction and reduced the viscosity of the homogenate. After gel filtration, there was no need for further reduction of the viscosity by the application of DNase (40). The cation exchange C 50 column using a linear gradient of 0.15 M NaCl and 0.5 M NaCl restricted the elution of the growth factor to one peak. In comparison this restriction failed in the purification of rh-bFGF (41). The higher molecular weight of α-ECGF and a different isoelectric point may be explanations for this observation. The growth factor was not stabilized with the zwitterionic detergent CHAPS (43) in order to prevent side-effects *in vivo*. The gradient in the heparin-sepharose affinity chromatography was found to be between 0.6 and 2 M NaCl. Whereas the elution of the main peak of FGF was described at a 1.4 M NaCl gradient (42), the ECGF peak was collected at a 2 M NaCl gradient. The polymyxine B column proved to be an efficient method to remove remaining endotoxin remnants, and the silver-stained gels showed no sign of a significant growth factor loss in polymyxine B column.

As a result of the animal experiments in rabbits and the results from the limulus lysate assay we were able to exclude the pyrogenic effects of ECGF. Thus there was no need to carry out high-pressure liquid chromatography (HPLC), which represents the main aspect of the purification of heparin-binding growth factors illustrated by the aforementioned authors (10,44). HPLC purification faces the problem that precipitations block the system (42). The main reason for omitting the HPLC procedure is the speculation that remnants of the TFA buffer used in HPLC induce inflammation and angiogenesis *in vivo* (29). Thus we sought to achieve sufficient purification without HPLC.

The production of recombinant human α-ECGF by our new combination of biotechnological methods proved to be a complex, but efficient method to produce the large quantities of growth factor required for clinical trials. From a 5-l culture medium (15 g bacteria) we purified, without HPLC, 200 mg of α-ECGF compared to the previous quantity of 60 mg aFGF reported by other authors using the HPLC methodology (42). The biological activity of ECGF was proved in the CAM assay and this result was confirmed by the increase of DNA synthesis in BHK 21 cells. Clinical trials in patients suffering from coronary heart disease who cannot be treated with conventional bypass surgery are currently under progress.

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