

Description

This invention relates to endothelial cell growth factor (ECGF), its recombinant DNA-directed synthesis, and ECGF's use in the treatment of endothelial cell damage and/or regeneration.

Endothelial cell growth factor, referred to herein as "ECGF", is a mitogen for endothelial cells *in vitro*. Growth of endothelial cells is a necessary step during the process of angiogenesis [Maciag, *Prog. Hemostasis and Thromb.*, 7:167-182 (1984); Maciag, T., Hoover, G.A., and Weinstein, R., *J. Biol. Chem.*, 257: 5333-5336 (1982)]. Bovine ECGF has been isolated by Maciag, et al., [*Science* 225:932-935 (1984)] using streptomycin sulfate precipitation, gel exclusion chromatography, ammonium sulfate precipitation and heparin-Sepharose® affinity chromatography. Bovine ECGF purified in this manner yields a single-chain polypeptide which possesses an anionic isoelectric point and an apparent molecular weight of 20,000 [Maciag, *supra*; Schreiber, et al., *J. Cell Biol.*, 101:1623-1626 (1985); and Schreiber, et al., *Proc. Natl. Acad. Sci.*, 82:6138-6142 (1985)]. More recently, multiple forms of bovine ECGF have been isolated by Burgess, et al., [*J. Biol. Chem.* 260:11389-11392 (1985)] by sodium chloride gradient elution of bovine ECGF from a heparin-Sepharose® column or by reversed-phase high pressure liquid chromatography (HPLC). The two isolated polypeptides, designated alpha- and beta-ECGF have apparent molecular weights of 17,000 and 20,000, respectively. Using this procedure, the bovine ECGF contained in 8,500 ml of bovine brain extract (6.25×10^7 total units) is concentrated into a total of 6 ml of alpha-ECGF (3.0×10^6 units) and 3 ml of beta-ECGF (5.2×10^5 units). This is a 9,300-fold purification of alpha-ECGF and 16,300-fold purification of beta-ECGF (Burgess, *supra*). Recently, murine monoclonal antibodies against bovine ECGF have been produced (Maciag, et al., *supra*) which may be useful in purifying bovine ECGF in a manner similar to the monoclonal antibody purification of Factor VIIIc described by Zimmerman and Fulcher in U.S. Patent No. 4,361,509.

The purification of two forms of endothelial cell growth factor (ECGF) from human brain has also been disclosed [Conn & Hatcher, *Biochem. Biophys. Res. Commun.*, vol. 24 pp 262-268 (1984)]. However, as was conceded by the same group in a later publication [Gimenez-Gallego et al., *Biochem. Biophys. Res. Commun.*, vol 135, pp 541-548 (1986)] the growth factors "purified" according to Conn & Hatcher were actually mixtures of peptides. One of these mixtures, "peak I", was shown by further purification and amino acid sequence analysis to contain two microheterogenous forms of acidic fibroblast growth factor as well as

an unidentified component. The other mixture, "peak II", was shown to contain two electrophoretically distinct forms of basic fibroblast growth factor.

Gimenez-Gallego et al., [*Science*, vol 230; pp 1385-1388, (1985)] disclose an acidic fibroblast growth factor a FGF derived from bovine brains and the amino acid sequences of two microheterogenous forms, namely a FGF-1 and a FGF-2.

In general, recombinant DNA techniques are known. See *Methods in Enzymology*. (Academic Press, New York) volumes 65 and 68 (1979); 100 and 101 (1983) and the references cited therein, all of which are incorporated herein by reference. An extensive technical discussion embodying most commonly used recombinant DNA methodologies can be found in Maniatis, et al., *Molecular Cloning*, Cold Spring Harbor Laboratory (1982). Genes coding for various polypeptides may be cloned by incorporating a DNA-fragment coding for the polypeptide in a recombinant DNA vehicle, e.g., bacterial or viral vectors, and transforming a suitable host. This host is typically an *Escherichia coli* (*E. coli*) strain, however, depending upon the desired product, eukaryotic hosts may be utilized. Clones incorporating the recombinant vectors are isolated and may be grown and used to produce the desired polypeptide on a large scale.

Several groups of workers have isolated mixtures of messenger RNA (mRNA) from eukaryotic cells and employed a series of enzymatic reactions to synthesize double-stranded DNA copies which are complementary to this mRNA mixture. In the first reaction, mRNA is transcribed into a single-stranded complementary DNA (cDNA) by an RNA-directed DNA polymerase, also called reverse transcriptase. Reverse transcriptase synthesizes DNA in the 5'-3' direction, utilizes deoxyribonucleoside 5'-triphosphates as precursors, and requires both a template and a primer strand, the latter of which must have a free 3'-hydroxyl terminus. Reverse transcriptase products, whether partial or complete copies of the mRNA template, often possess short, partially double-stranded hairpins ("loops") at their 3' termini. In the second reaction, these "hairpin loops" can be exploited as primers for DNA polymerases. Preformed DNA is required both as a template and as a primer in the action of DNA polymerase. The DNA polymerase requires the presence of a DNA strand having a free 3'-hydroxyl group, to which new nucleotides are added to extend the chain in the 5'-3' direction. The products of such sequential reverse transcriptase and DNA polymerase reactions still possess a loop at one end. The apex of the loop or "fold-point" of the double-stranded DNA, which has thus been created, is substantially a single-strand segment. In the third reaction, this single-strand segment is

cleaved with the single-strand specific nuclease S1 to generate a "blunt-end" duplex DNA segment. This general method is applicable to any mRNA mixture, and is described by Buell, et al., *J. Biol. Chem.*, **253**:2483 (1978).

The resulting double-stranded cDNA mixture (ds-cDNA) is inserted into cloning vehicles by any one of many known techniques, depending at least in part on the particular vehicle used. Various insertion methods are discussed in considerable detail in *Methods In Enzymology*, **68**:16-18 (1980), and the references cited therein.

Once the DNA segments are inserted, the cloning vehicle is used to transform a suitable host. These cloning vehicles usually impart an antibiotic resistance trait on the host. Such hosts are generally prokaryotic cells. At this point, only a few of the transformed or transfected hosts contain the desired cDNA. The sum of all transformed or transfected hosts constitutes a gene "library". The overall ds-cDNA library created by this method provides a representative sample of the coding information present in the mRNA mixture used as the starting material.

If an appropriate oligonucleotide sequence is available, it can be used to identify clones of interest in the following manner. Individual transformed or transfected cells are grown as colonies on a nitrocellulose filter paper. These colonies are lysed; the DNA released is bound tightly to the filter paper by heating. The filter paper is then incubated with a labeled oligonucleotide probe which is complementary to the structural gene of interest. The probe hybridizes with the cDNA for which it is complementary, and is identified by autoradiography. The corresponding clones are characterized in order to identify one or a combination of clones which contain all of the structural information for the desired protein. The nucleic acid sequence coding for the protein of interest is isolated and reinserted into an expression vector. The expression vector brings the cloned gene under the regulatory control of specific prokaryotic or eukaryotic control elements which allow the efficient expression (transcription and translation) of the ds-cDNA. Thus, this general technique is only applicable to those proteins for which at least a portion of their amino acid or DNA sequence is known for which an oligonucleotide probe is available. See, generally, Maniatis, et al., *supra*.

More recently, methods have been developed to identify specific clones by probing bacterial colonies or phage plaques with antibodies specific for the encoded protein of interest. This method can only be used with "expression vector" cloning vehicles since elaboration of the protein product is required. The structural gene is inserted into the vector adjacent to regulatory gene sequences that

control expression of the protein. The cells are lysed, either by chemical methods or by a function supplied by the host cell or vector, and the protein is detected by a specific antibody and a detection system such as enzyme immunoassay. An example of this is the lambda gt₁₁ system described by Young and Davis, *Proc. Nat'l. Acad. Sci. USA*, **80**:1194-1198 (1983) and Young and Davis, *Science*, **222**:778 (1983).

The present invention has made it possible to provide readily available, large quantities of ECGF or ECGF fragments. This has been achieved with oligonucleotides whose design was based upon knowledge of the amino acid sequence of bovine ECGF and which react specifically with the ECGF cDNA. Production of ECGF is achieved through the application of recombinant DNA technology to prepare cloning vehicles encoding the ECGF protein and procedures for recovering ECGF protein essentially free of other proteins of human origin.

Accordingly, the present invention provides ECGF or its fragments essentially free of other proteins of human origin. ECGF is produced by recombinant DNA techniques in host cells or other self-replicating systems and is provided in essentially pure form. The invention further provides replicable expression vectors incorporating a DNA sequence encoding ECGF and a self-replicating host cell system transformed or transfected thereby. The host system is usually of prokaryotic, e.g., *E. coli* or *B. subtilis*, or eukaryotic cells.

The ECGF is produced by a process which comprises (a) preparing a replicable expression vector capable of expressing the DNA sequence encoding ECGF in a suitable host cell system; (b) transforming said host system to obtain a recombinant host system; (c) maintaining said recombinant host system under conditions permitting expression of said ECGF-encoding DNA sequence to produce ECGF protein; and (d) recovering said ECGF protein, wherein said human endothelial cell growth factor has the sequence α -ECGF or β -ECGF as shown in Figure 8 of this application or is an allelic variation, modification or analogue of said α -ECGF or β -ECGF which retains biologically active properties of native ECGF.

Preferably, the ECGF-encoding replicable expression vector is made by preparing a ds-cDNA preparation representative of ECGF mRNA and incorporating the ds-cDNA into replicable expression vectors. The preferred mode of recovering ECGF comprises reacting the proteins expressed by the recombinant host system with a reagent composition comprising at least one binding step specific for ECGF. ECGF may be used as a therapeutic agent in the treatment of damaged or in regenerating blood vessels and other endothelial cell-lined structures.

Brief Description of the Drawings

Figure 1 illustrates a general procedure for enzymatic reactions to produce cDNA clones.

Figure 2 illustrates the production of a library containing DNA fragments inserted into lambda gt₁₁.

Figure 3 illustrates a partial amino acid sequence of bovine alpha and beta ECGF.

Line a: Amino-terminal amino acid sequence of bovine alpha ECGF.

Line b: Amino-terminal amino acid sequence of bovine beta ECGF. The portion in parenthesis corresponds to NH₂-terminal segment for which sequence could not be determined; amino acid composition is shown instead. The sequence beginning with PheAsn-Leu... was determined from trypsin-cleaved bovine beta ECGF.

Line c: Amino acid sequence of cyanogen bromide-cleaved bovine alpha ECGF.

Line d: Amino acid sequence of cyanogen bromide-cleaved bovine beta ECGF.

Figure 4 illustrates hydrogen-bonded base pairs.

Figure 5 illustrates the design of an oligonucleotide probe for human Endothelial Cell Growth Factor.

Figure 6 illustrates a schematic diagram of human ECGF cDNA clones 1 and 29. The open reading box represents the open reading frame encoding human beta ECGF. The EcoRI sites correspond to synthetic oligonucleotide linkers used in the construction of the cDNA library. The poly (A) tail at the 3' end of clone 1 is shown by A₁₇.

Figure 7 illustrates homology between human ECGF cDNA sequence and oligonucleotide probes.

Line a: Bovine trypsin- or cyanogen bromide-cleaved beta ECGF amino acid sequence.

Line b: Unique oligonucleotide probe.

Line c: Human ECGF cDNA sequence (determined from lambda ECGF clones 1 and 29).

Line d: Human ECGF amino acid sequence, deduced from cDNA sequence analysis.

Figure 8 illustrates the complete cDNA sequence of human ECGF. The cDNA inserts from ECGF clones 1 and 29 were subcloned into M13mp18 and the ECGF-encoding open reading frame and flanking regions sequenced by the chain termination method. In frame stop codons at the 5' and 3' ends of the ECGF-encoding open reading frame are indicated by the underlined sequence and trm, respectively. The single-letter notation for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu;

F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Figure 9 illustrates Northern blot analysis of ECGF mRNA. RNA was denatured in 2.2 M formaldehyde and 50% formamide and fractionated by electrophoresis in a 1.25% agarose gel containing 2.2 M formaldehyde. This was transferred to GeneScreen Plus (New England Nuclear) by blotting with 10X SSPE. Blots were hybridized to ³²p-labeled nick-translated probes of ECGF clone 1 at 65°C for 16 hours in a mixture containing 2X SSPE, 20X Denhardt's solution, yeast transfer RNA (200 ug/ml), and 0.2% SDS. The membrane was subsequently washed at 65°C, twice with 2X SSPE and 0.2% SDS, then twice with 0.2X SSPE and 0.2% SDS, air-dried, and exposed overnight to Kodak XAR film with an intensifying screen. The migration of 28S and 18S RNA is noted.

Lane 1: 10 ug human brain poly(A)-containing RNA.

Lane 2: 10 ug human adult liver poly(A)-containing RNA.

As used herein, "ECGF" denotes endothelial cell growth factor or its fragments produced by cell or cell-free culture systems, in bioactive forms having the capacity to influence cellular growth, differentiation, and migration *in vitro* as does ECGF native to the human angiogenic process.

Different alleles of ECGF may exist in nature. These variations may be characterized by differences in the nucleotide sequence of the structural gene coding for proteins of identical biological function. It is possible to produce analogs having single or multiple amino acid substitutions, deletions, additions, or replacements. All such allelic variations, modifications, and analogs resulting in derivatives of ECGF according to Fig. 8 which retain the biologically active properties of native ECGF are included within the scope of this invention.

"Expression vectors" refer to vectors which are capable of transcribing and translating DNA sequences contained therein, where such sequences are linked to other regulatory sequences capable of affecting their expression. These expression vectors must be replicable in the host organisms or systems either as episomes, bacteriophage, or as an integral part of the chromosomal DNA. One form of expression vector which is particularly suitable for use in the invention is the bacteriophage, viruses which normally inhabit and replicate in bacteria. Particularly desirable phage for this purpose are the lambda gt₁₀ and gt₁₁ phage described by Young and Davis, *supra*. Lambda gt₁₁ is a general recombinant DNA expression vector capable of producing polypeptides specified by the inserted DNA.

To minimize degradation, upon induction with a synthetic analogue of lactose (IPTG), foreign proteins or portions thereof are synthesized fused to the prokaryotic protein B-galactosidase. The use of host cells defective in protein degradation pathways may also increase the lifetime of novel proteins produced from the induced lambda gt₁₁ clones. Proper expression of foreign DNA in lambda gt₁₁ clones will depend upon the proper orientation and reading frame of the inserted DNA with respect to the B-galactosidase promoter and translation initiating codon.

Another form of expression vector useful in recombinant DNA techniques is the plasmid - a circular unintegrated (extra-chromosomal), double-stranded DNA loop. Any other form of expression vector which serves an equivalent function is suitable for use in the process of this invention.

Recombinant vectors and methodology disclosed herein are suitable for use in host cells covering a wide range of prokaryotic and eukaryotic organisms. Prokaryotic cells are preferred for the cloning of DNA sequences and in the construction of vectors. For example, *E. coli* K12 strain HB101 (ATCC No. 33894), is particularly useful. Of course, other microbial strains may be used. Vectors containing replication and control sequences which are derived from species compatible with the host cell or system are used in connection with these hosts. The vector ordinarily carries an origin of replication, as well as characteristics capable of providing phenotypic selection in transformed cells. For example, *E. coli* can be transformed using the vector pBR322, which contains genes for ampicillin and tetracycline resistance [Bolivar, et al., *Gene*, 2:95 (1977)].

These antibiotic resistance genes provide a means of identifying transformed cells. The expression vector may also contain control elements which can be used for the expression of the gene of interest. Common prokaryotic control elements used for expression of foreign DNA sequences in *E. coli* include the promoters and regulatory sequences derived from the B-galactosidase and tryptophan (trp) operons of *E. coli*, as well as the pR and pL promoters of bacteriophage lambda. Combinations of these elements have also been used (e.g., TAC, which is a fusion of the trp promoter with the lactose operator). Other promoters have also been discovered and utilized, and details concerning their nucleotide sequences have been published enabling a skilled worker to combine and exploit them functionally.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains

are commonly available. Yeast promoters suitable for the expression of foreign DNA sequences in yeast include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes. Suitable expression vectors may contain termination signals which provide for the polyadenylation and termination of the mRNA transcript of the cloned gene. Any vector containing a yeast-compatible promoter, origin of replication, and appropriate termination sequence is suitable for expression of ECGF.

Cell lines derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from a vertebrate or invertebrate source. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years. Examples of such useful hosts are the VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI38, BHK, COS-7, and MDCK cell lines. Expression vectors for such cells ordinarily include an origin of replication, a promoter located in front of the gene to be expressed, RNA splice sites (if necessary), and transcriptional termination sequences.

For use in mammalian cells, the control functions (promoters and enhancers) on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently, Simian Virus 40 (SV40). Eukaryotic promoters, such as the promoter of the murine metallothionein gene [Paulakis and Hamer, *Proc. Natl. Acad. Sci.* 80:397-401 (1983)], may also be used. Further, it is also possible, and often desirable, to utilize the promoter or control sequences which are naturally associated with the desired gene sequence, provided such control sequences are compatible with the host system. To increase the rate of transcription, eukaryotic enhancer sequences can also be added to the construction. These sequences can be obtained from a variety of animal cells or oncogenic retroviruses such as the mouse sarcoma virus.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as that provided by SV40 or other viral sources, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Host cells can prepare ECGF which can be of a variety of chemical compositions. The protein is produced having methionine as its first amino acid. This methionine is present by virtue of the ATG start codon naturally existing at the origin of the structural gene or by being engineered before a segment of the structural gene. The protein may also be intracellularly or extracellularly cleaved, giv-

ing rise to the amino acid which is found naturally at the amino terminus of the protein. The protein may be produced together with either its own or a heterologous signal peptide, the signal polypeptide being specifically cleavable in an intra- or extracellular environment. Finally, ECGF may be produced by direct expression in mature form without the necessity of cleaving away any extraneous polypeptide.

Recombinant host cells refer to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, ECGF is produced as a consequence of this transformation. ECGF or its fragments produced by such cells are referred to as "recombinant ECGF".

The procedures below are but some of a wide variety of well established procedures to produce specific reagents useful in the process of this invention. The general procedure for obtaining an mRNA mixture is to obtain a tissue sample or to culture cells producing the desired protein, and to extract the RNA by a process such as that disclosed by Chirgwin, et al., *Biochemistry*, 18:5294 (1979). The mRNA is enriched by poly(A)mRNA-containing material by chromatography on oligo (dT) cellulose or poly(U) Sepharose, followed by elution of the poly(A) containing mRNA fraction.

The above poly(A) containing mRNA-enriched fraction is used to synthesize a single-strand complementary cDNA (ss-cDNA) using reverse transcriptase. As a consequence of DNA synthesis, a hairpin loop is formed at the 3' end of the DNA which will initiate second-strand DNA synthesis. Under appropriate conditions, this hairpin loop is used to effect synthesis of the ds-cDNA in the presence of DNA polymerase and deoxyribonucleotide triphosphates.

The resultant ds-cDNA is inserted into the expression vector by any one of many known techniques. In general, methods can be found in Maniatis, et al., *supra*, and *Methods In Enzymology*, Volumes 65 and 68 (1980); and 100 and 101 (1983). In general, the vector is linearized by at least one restriction endonuclease, which will produce at least two blunt or cohesive ends. The ds-cDNA is ligated with or joined into the vector insertion site.

If prokaryotic cells or other cells which contain substantial cell wall material are employed, the most common method of transformation with the expression vector is calcium chloride pretreatment as described by Cohen, R.N., et al., *Proc. Nat'l. Acad. Sci. USA*, 69:2110 (1972). If cells without cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method described by Graham and Van der Eb, *Virology*, 52:456 (1973). Other methods for intro-

ducing DNA into cells such as nuclear injection, viral infection or protoplast fusion may be successfully used. The cells are then cultured on selective media, and proteins for which the expression vector encodes are produced.

Clones containing part or the entire cDNA for ECGF are identified with specific oligonucleotide probes deduced from a partial amino acid sequence determination of ECGF. This method of identification requires that the non-degenerate oligonucleotide probe be designed such that it specifically hybridizes to ECGF ds-cDNA. Clones containing ECGF cDNA sequences are isolated by radioactively labeling the oligonucleotide probe with ³²P-ATP, hybridizing the radioactive oligonucleotide probe to the DNA of individual clones of a cDNA library containing ECGF-cDNA, and detection and isolation of the clones which hybridize by autoradiography. Such a cloning system is applicable to the lambda gt₁₁ system described by Young and Davis, *supra*.

Clones containing the entire sequence of ECGF are identified using as probe the cDNA insert of the ECGF recombinants isolated during the initial screening of the recombinant lambda gt₁₁ cDNA library with ECGF-specific oligonucleotides. Nucleotide sequencing techniques are used to determine the sequence of amino acids encoded by the cDNA fragments. This information may be used to determine the identity of the putative ECGF cDNA clones by comparison to the known amino acid sequence of the amino-terminus of bovine ECGF and of a peptide derived by cyanogen bromide cleavage of ECGF.

EXAMPLE

A. Preparation of Total RNA

Total RNA (messenger, ribosomal and transfer) was extracted from fresh two-day old human brain stem essentially as described by Chirgwin, *supra*, (1979). Cell pellets were homogenized in 5 volumes of a solution containing 4 M guanidine thiocyanate, and 25 mM Antifoam A (Sigma Chemical Co., St. Louis, Mo.). The homogenate was centrifuged at 6,000 rpm in a Sorvall GSA rotor for 15 minutes at 10°C. The supernatant fluid was adjusted to pH 5.0 by addition of acetic acid and the RNA precipitated by 0.75 volumes of ethanol at -20°C for two hours. RNA was collected by centrifugation and dissolved in 7.5 M guanidine hydrochloride containing 2 mM sodium citrate and 5 mM dithiothreitol. Following two additional precipitations using 0.5 volumes of ethanol, the residual guanidine hydrochloride was extracted from the precipitate with absolute ethanol. RNA was dissolved in sterile water, insoluble material removed

by centrifugation, and the pellets were re-extracted with water. The RNA was adjusted to 0.2 M potassium acetate and precipitated by addition of 2.5 volumes of ethanol at -20 °C overnight.

B. Preparation of Poly(A)-containing RNA

The total RNA precipitate, prepared as described above, was dissolved in 20 mM Hepes buffer (pH 7.2) containing 10 mM EDTA and 1% SDS, heated at 65 °C for 10 minutes, then quickly cooled to 25 °C. The RNA solution was then diluted with an equal volume of water, and NaCl was added to bring the final concentration to 300 mM NaCl. Samples containing up to 240 A₂₆₀ units of RNA were chromatographed on poly(U)-Sephadex® using standard procedures. Poly(A)-containing RNA was eluted with 70% formamide containing 1 mM Hepes buffer (pH 7.2), and 2 mM EDTA. The eluate was adjusted to 0.24 M NaCl and the RNA was precipitated by 2.5 volumes of ethanol at -20 °C.

C. Construction of cDNA Clones in Lambda gt₁₁

The procedure followed for the enzymatic reaction is shown in Figure 1. The mRNA (20 µg) was copied into ds-cDNA with reverse transcriptase and DNA polymerase I exactly as described by Buell, et al., *supra*, and Wilkensen, et al., *J. Biol. Chem.*, 253:2483 (1978). The ds-cDNA was desalted on Sephadex® G-50 and the void-volume fractions further purified on an Elutip-D® column (Schleicher & Schuell, Keene, NH) following the manufacturer's directions. The ds-cDNA was made blunt-ended by incubation with SI nuclease [Ricca, et al., *J. Biol. Chem.*, 256:10362 (1981)]. The reaction mixture consisted of 0.2 M sodium acetate (pH 4.5), 0.4 M sodium chloride, 2.5 mM zinc acetate and 0.1 unit of SI nuclease per ng of ds-cDNA, made to a final reaction volume of 100 µl. The ds-cDNA was incubated at 37 °C for one hour, extracted with phenol:chloroform, and then desalted on a Sephadex® G-50 column as described above.

The ds-cDNA was then treated with EcoRI methylase and Klenow fragment of DNA polymerase I using reaction conditions described in Maniatis, et al., *Molecular Cloning*, *supra*. The cDNA was again desalted on Sephadex® G-50 as described above and then ligated to 0.5 µg of phosphorylated EcoRI linkers using T₄ DNA ligase (Maniatis, et al., *supra*). The mixture was cleaved with EcoRI and fractionated on an 8% acrylamide gel in Trisborate buffer (Maniatis, et al., *supra*). DNA with a size greater than 1 kilobase was eluted from the gel and recovered by binding to an Elutip-D® column, eluted with 1 M NaCl and then collected by ethanol precipitation.

As shown in Figure 2, the DNA fragments were then inserted into EcoRI cleaved and phosphatase-treated lambda gt₁₁, using T₄ DNA ligase. A library of 5.7 x 10⁶ phage was produced, of which approximately 65% were recombinant phage. The library was amplified by producing plate stocks at 42 °C on *E. coli* Y1088 [supE supF metB trpR hsdR⁻ hsdM⁺ tonA21 strA lacU169 (proC::Tn5) (pMC9)]. Amplification procedures are described in Maniatis, et al., *supra*. Important features of this strain, described by Young and Davis, *supra*, include (1) supF (required suppression of the phage amber mutation in the S gene), (2) hsdR⁻ hsdM⁺ - (necessary to prevent restriction of foreign DNA prior to host modification), and (3) lacU169 (proC::Tn5), and (4) (pMC9) (a lac I-bearing pBR322 derivative which represses, in the absence of an inducer, the expression of foreign genes that may be detrimental to phage and/or cell growth).

D. Identification of Clones Containing ECGF Sequence

To screen the library for recombinant phage containing ECGF cDNA, 1.5 x 10⁶ phage were plated on a lawn of *E. coli* Y1090 [ΔlacU169 proΔ lon araD139 strA supF (trpC22::Tn10) (pMC9)] and incubated at 42 °C for 6 hours. After the plates were refrigerated overnight, a nitrocellulose filter was overlaid on the plates. The position of the filter was marked with a needle. The filter removed after one minute and left to dry at room temperature. From each plate, a duplicate filter was prepared exactly as described, except that the filter was left in contact with the plate for 5 minutes. All filters were then prepared for hybridization, as described in Maniatis, et al., *supra*. This involved DNA denaturation in 0.5 M NaOH, 1.5 M NaCl, neutralization in 1 M Tris-HCl, pH 7.5, 1.5 M NaCl, and heating of the filters for 2 hours at 80 °C *in vacuo*.

To screen the human brain stem cDNA library for clones containing ECGF inserts, a specific oligonucleotide was designed. This oligonucleotide was based upon a partial amino acid sequence analysis of the amino terminus of ECGF. As shown in Figure 3, lines a & b, bovine ECGF is isolated as two species, designated alpha and beta ECGF, which differ only in the amino acids found at the respective amino termini. As shown in Figure 3, line b, beta-ECGF is a slightly larger species than alpha-ECGF. The exact amino acid sequence at the amino terminus of beta-ECGF is undetermined, however, a sequence derived from fast atom bombardment mass spectral analysis and the amino acid composition of the amino terminal tryptic peptide of bovine beta-ECGF is shown. The amino terminal blocking group appears to be acetyl. If intact beta-ECGF is cleaved by trypsin, a second

amino amino acid sequence found in beta but not alpha ACGF starting with PheAsnLeu... is determined. This sequence is also found at the amino terminus of acidic fibroblast growth factor [Thomas, K.A. et al., *Proc. Natl. Acad. Sci.*, **82**:6409-6413 (1985)]. The amino terminus of alpha-ECGF is Asn-TyrLys... (Figure 3, line a) and is the equivalent of beta-ECGF minus an amino terminal extension. In Figure 3, lines c and d set forth for comparison the amino acid sequence of cyanogen bromide-cleaved bovine alpha and beta ECGF, respectively.

For oligonucleotide design, the amino acid sequence IleLeuProAspGlyThrValAspGlyThrLys, corresponding to alpha-ECGF amino acids 19-29 inclusive, was chosen. Rather than design a mixture of oligonucleotides covering all of the possible coding sequences (owing to the degeneracy of the genetic code), a long unique oligonucleotide was designed. Such oligonucleotide probes have been previously shown to be successful probes in screening complex cDNA [Jaye, et al., *Nucleic Acids Research* **11**:2325-2335, (1983)] and genomic [Gitschier, et al., *Nature*, **312**:326-330 (1984)] libraries. Three criteria were used in designing the ECGF probe: (1) The dinucleotide CG was avoided. This strategy was based upon the observed under representation of the CG dinucleotide in eukaryotic DNA [Josse, et al., *J. Biol. Chem.* **236**:864-875, (1961)]; (2) preferred codon utilization data was used wherever possible. A recent and comprehensive analysis of human codon utilization was found in Lathe, *J. Mol. Biol.* **183**:1-12 (1985); and (3) wherever the strategies of CG dinucleotide and preferred codon utilization were uninformative, unusual base pairing was allowed. This strategy was based upon the natural occurrence of G:T, I:T, I:A and I:C base pairs which occur in the interaction between tRNA anticodons and mRNA codons [Crick, *J. Mol. Biol.* **19**:548-555, (1966)]. A diagram of usual and unusual base pairs is shown in Figure 4. Use of I (Inosine) in a hybridization probe was first demonstrated, in a model experiment, by Ohtsuka, et al., *J. Biol. Chem.* **260**:2605-2608 (1985). The overall strategy and choice made in the design of the oligonucleotide used to screen the human brain stem cDNA library for ECGF is shown in Figure 5. In addition, two other oligonucleotides, designed with the same strategy, were constructed.

Approximately 30 pmole of the oligonucleotide shown in Figure 5 were radioactively labeled by incubation with ³²P-gamma-ATP and T4 polynucleotide kinase, essentially as described by Maniatis, et al., *supra*. Nitrocellulose filters, prepared as described above, were prehybridized at 42°C in 6X SSPE (1X SSPE = 0.18M NaCl, 0.01M NaHPO₄ pH 7.2, 0.001M EDTA), 2X Denhardt's (1X Denhardt's = 0.02% each Ficoll®, polyvinylpyr-

olidone, bovine serum albumin), 5% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. The ³²P-labeled oligonucleotide was added following four hours of prehybridization, and hybridization continued overnight at 42°C. Unhybridized probe was removed by sequential washing at 37°C in 2X SSPE, 0.1% SDS.

From 1.5 x 10⁶ plaques screened, 2 plaques gave positive autoradiographic signals after overnight exposure. These clones were purified to homogeneity by repeated cycles of purification using the above oligonucleotide as hybridization probe.

The two clones that were isolated, ECGF clones 1 and 29, were analyzed in further detail. Upon digestion with EcoRI, clone 1 and 29 revealed cDNA inserts of 2.2 and 0.3 Kb, respectively. Nick translation of cloned cDNA and its subsequent use as a radiolabeled probe in Southern blot analysis (Maniatis, et al., *supra*) revealed that clones 1 and 29 were related and overlapping clones. The overlapping nature of these two clones is shown in Figure 6.

Clones 1 and 29 were analyzed in further detail as follows: An additional two oligonucleotides were designed, based upon the amino acid sequence of bovine ECGF. These oligonucleotides were designed based upon the same considerations as those used in the design of the oligonucleotide used to isolate clones 1 and 29. These oligonucleotides (ECGF oligonucleotides II and III) are shown in Figure 7. These two oligonucleotides as well as oligo(dT)₁₈ were radioactively labeled in a kination reaction as described above and used as hybridization probes in Southern blotting experiments. The results of these experiments showed that the 0.3 Kb cDNA insert of clone 29 hybridized to ECGF oligonucleotides I and II but not to ECGF oligonucleotide III or oligo(dT)₁₈; the 2.2 Kb cDNA insert of clone 1 hybridized to oligonucleotide I, II, III as well as oligo(dT)₁₈. These data and subsequent nucleotide sequence determination of clones 1 and 29 showed that the 3' end of clone 1 ends with a poly(A) tail. Hybridization of clone 1 to ECGF oligonucleotide III, which is based on a cyanogen bromide cleavage product of bovine ECGF, as well as to oligo(dT)₁₈, strongly suggested that this clone contains the rest of the coding sequence for both alpha and beta ECGFs as well as a large (greater than 1 Kb) 3' flanking sequence.

The cDNA inserts from clones 1 and 29 were isolated, subcloned into M13mp18, and the ECGF-encoding open reading frame and flanking regions sequenced by the chain termination method [Sanger et al., *Proc. Natl. Acad. Sci. USA* **74**:5463-5467 (1977)]. The nucleotide sequence of these clones and the amino acid sequence deduced from the nucleic acid sequence is shown in Figure 8. Examination of the nucleotide sequence reveals an open

reading frame of 465 nucleotides encoding human ECGF. The 155 amino acids of human ECGF were found to be flanked by translation stop codons. The NH₂-terminal amino acid of human beta ECGF deduced from the cDNA sequence is methionine, which most likely serves as the translation initiation residue. These data, together with the relatively non-hydrophobic nature of the first 15-20 amino terminal residues, strongly suggest that human beta ECGF is synthesized without a NH₂-terminal signal peptide. A comparison of Figures 3 and 8 shows that the amino terminal amino acid sequence of trypsin-cleaved bovine beta ECGF as well as that of bovine alpha ECGF are nearly identical to the amino acid sequence predicted from the nucleotide sequence of lambda ECGF clones 1 and 29. An overall homology between the two species of over 95% is observed.

Northern blot analysis (Maniatis, et al, *supra*) reveals that ECGF mRNA is a single molecular species which comigrates with 28S rRNA (Figure 9). Considering the variation in the estimated size of 28S rRNA, the approximate size of ECGF mRNA is 4.8 ± 1.4 Kb. All of the sequence encoding the mature forms of both alpha and beta ECGF is encoded within ECGF clones 1 and 29, which together encompasses approximately 2.3 Kb. Thus, these data demonstrate that the region 5' and flanking the ECGF-encoding sequences, is very large (approximately 2.5 ± 1.4 Kb).

cDNA inserts from clone 1 and clone 29 were excised by digestion with EcoRI and subcloned in pUC8 at the EcoRI site. The plasmid formed from clone 1 was designated pDH15 and the plasmid formed from clone 29 was designated pDH14. The plasmids were deposited in American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. The plasmid from clone 1, pDH15, was designated ATCC 53336 and the plasmid from clone 29, pDH 14, was designated ATCC 53335.

Thus, this example describes experimental procedures which provide human endothelial cell growth factor essentially free of other proteins of human origin.

ECGF has utility in the growth and amplification of endothelial cells in culture. Currently, ECGF for cell culture use is extracted from bovine brain by the protocol of Maciag, et al., [*Proc. Natl. Acad. Sci.*, 76:11, 5674-5678 (1978)]. This crude bovine ECGF is mitogenic for human umbilical vein endothelial cells [Maciag, et al., *J. Biol. Chem.* 257:5333-5336 (1982)] and endothelial cells from other species. Utilization of heparin with ECGF and a fibronectin matrix permits the establishment of stable endothelial cell clones. The recommended concentration of this crude bovine ECGF for use as a mitogen *in vitro* is 150 micrograms per milliliter of growth medium.

Recombinant DNA-derived human ECGF has utility, therefore, as an improved substitute for crude bovine ECGF in the *in vitro* culturing of human endothelial cells and other mesenchymal cells for research use. The activity of human ECGF is expected to be the same as or better than bovine ECGF in the potentiation of endothelial cell growth due to the high degree of homology in the amino acid sequences of both proteins. The expected effective dose range for potentiating cell division and growth *in vitro* is 5 - 10 ng of purified ECGF per milliliter of culture medium. Production of the ECGF via recombinant-DNA technologies as outlined in this patent application and subsequent purification as described by Burgess, et al., [*J. Biol. Chem.* 260:11389-11392 (1985)] will provide large quantities of a pure product of human origin (heretofore unavailable in any quantity or purity) with which to develop models of human homeostasis and angiogenesis.

Recombinant DNA-derived human ECGF also has utility in the potentiation of cell growth on a prosthetic device, rather than a tissue culture flask or bottle. This device may or may not be coated with other molecules which would facilitate the attachment of endothelial cells to the device. These facilitating molecules may include extracellular matrix proteins (eg. fibronectin, laminin, or one of the collagens), human serum albumin, heparin or other glycosaminoclycans or inert organic molecules. Endothelial cells would be cultured on these surfaces using effective doses of ECGF in the culture medium, ultimately covering the device with an endothelial cell monolayer. This device would then provide a non-thrombogenic surface on the prosthetic device, thus reducing the risk of potentially life-threatening thrombogenic events subsequent to implantation of the prosthetic device.

ECGF has utility in diagnostic applications. Schreiber, et al., [*Proc. Natl. Acad. Sci.* 82:6138 (1985)] developed a double antibody immunoassay for bovine ECGF. In this assay, 96-well polyvinyl chloride plates were coated with rabbit anti-ECGF and the remaining binding sites subsequently blocked with 10% normal rabbit serum. Samples of ECGF were then added to the wells and incubated. After washing, murine monoclonal anti-ECGF was added. After incubation and several washes, rabbit anti-mouse IgG coupled with peroxidase was added. The reaction product was quantitated spectrophotometrically after conversion of O-phenylenediamine in the presence of hydrogen peroxide. A similarly constructed immunoassay may be useful for monitoring human ECGF levels in disease states affecting endothelial cell growth. Purified recombinant-DNA derived ECGF would be useful as a standard reagent in quantifying unknown ECGF samples.

ECGF also may have potential in the treatment of damaged or in the regeneration of blood vessels and other endothelial cell-lined structures.

It should be appreciated that the present invention is not to be construed as being limited by the illustrative embodiment. It is possible to produce still other embodiments without departing from the inventive concepts herein disclosed. Such embodiments are within the ability of those skilled in the art.

Claims

Claims for the following Contracting States :
BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. A process for producing human endothelial cell growth factor comprising, providing a replicable expression vector capable of expressing the DNA sequence encoding human endothelial cell growth factor in a suitable host, transforming said host to obtain a recombinant host, and maintaining said recombinant host under conditions permitting expression of said endothelial cell growth factor - encoding cDNA sequence to produce endothelial cell growth factor, said human endothelial cell growth factor having the sequence of α -ECGF or β -ECGF shown in Figure 8 or being an allelic variant, modification or analogue of said α -ECGF or β -ECGF, which retains the biologically active properties of native ECGF.
2. The process according to claim 1 including the further step of recovering said endothelial cell growth factor.
3. The process according to claim 2 wherein said expression vector is a bacteriophage.
4. The process according to claim 3 wherein said bacteriophage is a member of the group consisting of lambda gt₁₀ and lambda gt₁₁.
5. The process according to claim 2 wherein said expression vector is a plasmid.
6. The process according to claim 5 wherein said plasmid is derived from pBR322.
7. The process according to claim 2 wherein the control function on said expression vector is provided by viral material.
8. The process according to claim 7 wherein said viral material is derived from a member of the group consisting of bovine papilloma virus, Epstein Barr virus, adenovirus, Simian virus 40 and baculovirus.
9. Endothelial cell growth factor obtainable by the process of claim 2.
10. A replicable expression vector capable of expressing endothelial cell growth factor as defined in claim 9 in a self-replicating recombinant system.
11. A self-replicating recombinant system transformed with the vector of claim 10.
12. The recombinant system according to claim 11 wherein said system is in a cell.
13. The recombinant system according to claim 11 wherein said system is cell-free.
14. The recombinant system according to claim 11 obtained by transforming or infecting a member of the group consisting of an E. coli, B. subtilis, insect, yeast and vertebrate cell.
15. The recombinant system according to claim 11 obtained by transforming eukaryotes.
16. The process according to claim 1 wherein recovering said endothelial cell growth factor comprises reaction of the proteins expressed by the recombinant host system with a reagent composition comprising at least one binding protein specific for endothelial cell growth factor.
17. Human endothelial cell growth factor having the sequence of α -ECGF or β -ECGF shown in Figure 8, or being an allelic variant, modification or analogue of said α -ECGF or β -ECGF, which retains the biologically active properties of native ECGF, said human endothelial cell growth factor being essentially free of other proteins of human origin.
18. The endothelial cell growth factor of claim 17 comprising a polypeptide sequence extending from the NH₂-terminal amino acid of endothelial cell growth factor.
19. A cDNA clone comprising the complete coding sequence for human endothelial cell growth factor, said cDNA having the DNA sequence encoding α -ECGF or β -ECGF shown in Figure 8 or being an allelic variant, modification or analogue of said DNA sequence which encodes a protein retaining the biologically active properties of native ECGF.
20. The cDNA clone of claim 19 containing untranslated nucleotide sequences located 5' and 3'



ECGF also may have potential in the treatment of damaged or in the regeneration of blood vessels and other endothelial cell-lined structures.

It should be appreciated that the present invention is not to be construed as being limited by the illustrative embodiment. It is possible to produce still other embodiments without departing from the inventive concepts herein disclosed. Such embodiments are within the ability of those skilled in the art.

Claims

Claims for the following Contracting States :
BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. A process for producing human endothelial cell growth factor comprising, providing a replicable expression vector capable of expressing the DNA sequence encoding human endothelial cell growth factor in a suitable host, transforming said host to obtain a recombinant host, and maintaining said recombinant host under conditions permitting expression of said endothelial cell growth factor - encoding cDNA sequence to produce endothelial cell growth factor, said human endothelial cell growth factor having the sequence of α -ECGF or β -ECGF shown in Figure 8 or being an allelic variant, modification or analogue of said α -ECGF or β -ECGF, which retains the biologically active properties of native ECGF.
2. The process according to claim 1 including the further step of recovering said endothelial cell growth factor.
3. The process according to claim 2 wherein said expression vector is a bacteriophage.
4. The process according to claim 3 wherein said bacteriophage is a member of the group consisting of lambda gt₁₀ and lambda gt₁₁.
5. The process according to claim 2 wherein said expression vector is a plasmid.
6. The process according to claim 5 wherein said plasmid is derived from pBR322.
7. The process according to claim 2 wherein the control function on said expression vector is provided by viral material.
8. The process according to claim 7 wherein said viral material is derived from a member of the group consisting of bovine papilloma virus, Epstein Barr virus, adenovirus, Simian virus 40 and baculovirus.
9. Endothelial cell growth factor obtainable by the process of claim 2.
10. A replicable expression vector capable of expressing endothelial cell growth factor as defined in claim 9 in a self-replicating recombinant system.
11. A self-replicating recombinant system transformed with the vector of claim 10.
12. The recombinant system according to claim 11 wherein said system is in a cell.
13. The recombinant system according to claim 11 wherein said system is cell-free.
14. The recombinant system according to claim 11 obtained by transforming or infecting a member of the group consisting of an E. coli, B. subtilis, insect, yeast and vertebrate cell.
15. The recombinant system according to claim 11 obtained by transforming eukaryotes.
16. The process according to claim 1 wherein recovering said endothelial cell growth factor comprises reaction of the proteins expressed by the recombinant host system with a reagent composition comprising at least one binding protein specific for endothelial cell growth factor.
17. Human endothelial cell growth factor having the sequence of α -ECGF or β -ECGF shown in Figure 8, or being an allelic variant, modification or analogue of said α -ECGF or β -ECGF, which retains the biologically active properties of native ECGF, said human endothelial cell growth factor being essentially free of other proteins of human origin.
18. The endothelial cell growth factor of claim 17 comprising a polypeptide sequence extending from the NH₂-terminal amino acid of endothelial cell growth factor.
19. A cDNA clone comprising the complete coding sequence for human endothelial cell growth factor, said cDNA having the DNA sequence encoding α -ECGF or β -ECGF shown in Figure 8 or being an allelic variant, modification or analogue of said DNA sequence which encodes a protein retaining the biologically active properties of native ECGF.
20. The cDNA clone of claim 19 containing untranslated nucleotide sequences located 5' and 3'



to the coding sequence of human endothelial cell growth factor as defined by the endothelial cell growth factor mRNA.

21. A composition comprising a therapeutically effective amount of human endothelial cell growth factor as defined in claim 17 or 18 to promote wound healing in a mixture with an acceptable carrier.

Claims for the following Contracting State : AT

1. A process for producing human endothelial cell growth factor comprising, providing a replicable expression vector capable of expressing the DNA sequence encoding human endothelial cell growth factor in a suitable host, transforming said host to obtain a recombinant host, and maintaining said recombinant host under conditions permitting expression of said endothelial cell growth factor encoding cDNA sequence to produce endothelial cell growth factor, said human endothelial cell growth factor having the sequence of α -ECGF or β -ECGF shown in Figure 8 or being an allelic variant, modification or analogue of said α -ECGF or β -ECGF, which retains the biologically active properties of native ECGF.
2. The process according to claim 1 including the further step of recovering said endothelial cell growth factor.
3. The process according to claim 2 wherein said expression vector is a bacteriophage.
4. The process according to claim 3 wherein said bacteriophage is a member of the group consisting of lambda gt₁₀ and lambda gt₁₁.
5. The process according to claim 2 wherein said expression vector is a plasmid.
6. The process according to claim 5 wherein said plasmid is derived from pBR322.
7. The process according to claim 2 wherein the control function on said expression vector is provided by viral material.
8. The process according to claim 7 wherein said viral material is derived from a member of the group consisting of bovine papilloma virus, Epstein Barr virus, adenovirus, Simian virus 40 and baculovirus.
9. A method for preparing a replicable expression vector capable of expressing endothelial cell

growth factor as obtainable by any of claim 1 to 8 in a self-replicating recombinant system, wherein a DNA insert coding for ECGF as obtainable according to any of claims 1 to 8 is introduced into a replicable expression vector by recombinant DNA technology.

10. A method for preparing a self-replicating recombinant system expressing an endothelial cell growth factor as obtainable by any of claim 1 to 8, wherein a self-replicating system is transformed with a vector obtainable according to claim 9.

11. The method according to claim 10, wherein said system is in a cell.

12. The method according to claim 10, wherein said system is cell free.

13. The method according to claim 10, wherein a member of the group consisting of an *E.coli*, *B. subtilis*, insect, yeast and vertebrate cell is transformed or infected.

14. The method according to claim 10, wherein a eukaryotic organism is transformed.

15. The process according to claim 1, wherein recovering said endothelial cell growth factor comprises reaction of the proteins expressed by the recombinant host system with a reagent composition comprising at least one binding protein specific for endothelial cell growth factor.

16. The process according to claim 2, wherein said human endothelial cell growth factor is essentially free of other proteins of human origin.

17. The process according to claim 16, wherein said endothelial cell growth factor comprises a polypeptide sequence extending from the NH₂-terminal amino acid of endothelial cell growth factor.

18. A method for preparing a cDNA clone comprising the complete coding sequence for human endothelial cell growth factor, said cDNA having the DNA sequence encoding α -ECGF or β -ECGF shown in Figure 8 or being an allelic variant, modification or analogue of said DNA sequence which encodes a protein retaining the biologically active properties of native ECGF, wherein said cDNA clone is prepared in a manner known per se.



19. The method according to claim 18, wherein the cDNA clone contains untranslated nucleotide sequences located 5' and 3' to the coding sequence of human endothelial cell growth factor as defined by the endothelial cell growth factor mRNA.

20. A method for preparing a composition for promotion of wound healing, comprising combining a therapeutically effective amount of human endothelial cell growth factor as obtainable in any of claims 1 to 8 and an acceptable carrier.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. Verfahren zum Erzeugen von humanem Endothelzellwachstumsfaktor, umfassend das Bereitstellen eines replikationsfähigen Expressionsvektors, der zur Expression der DNA-Sequenz, die für humanen Endothelzellwachstumsfaktor kodiert, in einem geeigneten Wirt fähig ist, das Transformieren des Wirtes zum Erhalten eines rekombinanten Wirtes und das Halten des rekombinanten Wirtes unter Bedingungen, die die Expression der für den Endothelzellwachstumsfaktor kodierenden cDNA-Sequenz erlauben, um Endothelzellwachstumsfaktor zu erzeugen, wobei der humane Endothelzellwachstumsfaktor die Sequenz von α -ECGF oder β -ECGF hat, die in Figur 8 gezeigt ist, oder eine allelische Variante, eine Modifikation oder ein Analog des α -ECGFs oder β -ECGFs ist, das die biologisch aktiven Eigenschaften von nativem ECGF beibehält.

2. Verfahren nach Anspruch 1 einschließlich des weiteren Schritts der Wiedergewinnung des Endothelzellwachstumsfaktors.

3. Verfahren nach Anspruch 2, worin der Expressionsvektor ein Bakteriophage ist.

4. Verfahren nach Anspruch 3, worin der Bakteriophage ein Mitglied der aus Lambda gt₁₀ und Lambda gt₁₁ bestehenden Gruppe ist.

5. Verfahren nach Anspruch 2, worin der Expressionsvektor ein Plasmid ist.

6. Verfahren nach Anspruch 5, worin das Plasmid von pBR322 abgeleitet ist.

7. Verfahren nach Anspruch 2, worin die Kontrollfunktion auf dem Expressionsvektor durch virales Material bereitgestellt wird.

8. Verfahren nach Anspruch 7, worin das virale Material von einem Mitglied der aus Rinderpapillomavirus, Epstein-Barr-Virus, Adenovirus, Affenvirus 40 (SV40) und Bakulovirus bestehenden Gruppe abgeleitet ist.

9. Endothelzellwachstumsfaktor, erhältlich durch das Verfahren nach Anspruch 2.

10. Replizierbarer Expressionsvektor, der zur Expression von Endothelzellwachstumsfaktor, wie definiert in Anspruch 9, in einem selbstreplizierenden rekombinanten System fähig ist.

11. Selbstreplizierendes rekombinantes System, transformiert mit dem Vektor nach Anspruch 10.

12. Rekombinantes System nach Anspruch 11, worin das System in einer Zelle ist.

13. Rekombinantes System nach Anspruch 11, worin das System zellfrei ist.

14. Rekombinantes System nach Anspruch 11, das durch Transformieren oder Infizieren eines Mitgliedes der aus *E. coli*, *B. subtilis*, Insekten, Hefe oder Vertebratenzellen bestehenden Gruppe erhältlich ist.

15. Rekombinantes System nach Anspruch 11, erhalten durch Transformation von Eukaryonten.

16. Verfahren nach Anspruch 1, worin das Wiedergewinnen des Endothelzellwachstumsfaktors die Reaktion der von dem rekombinanten Wirtssystem exprimierten Proteine mit einer Reagenzzusammensetzung umfaßt, die mindestens ein Bindeprotein umfaßt, das für Endothelzellwachstumsfaktor spezifisch ist.

17. Humaner Endothelzellwachstumsfaktor mit der Sequenz von α ECGF oder β -ECGF, gezeigt in Figur 8, oder eine allelische Variante, eine Modifikation oder ein Analog des α -ECGF oder β -ECGF, das die biologisch aktiven Eigenschaften von nativem ECGF beibehält, wobei der humane Endothelzellwachstumsfaktor im wesentlichen frei von anderen Proteinen humanen Ursprungs ist.

18. Endothelzellwachstumsfaktor nach Anspruch 17, umfassend eine Polypeptidsequenz, die sich von der NH₂-terminalen Aminosäure des Endothelzellwachstumsfaktors erstreckt.

19. cDNA-Klon, umfassend die vollständige kodierende Sequenz für humanen Endothelzell-



wachstumsfaktor, wobei die cDNA eine DNA-Sequenz hat, die für α -ECGF oder β -ECGF, gezeigt in Figur 8, kodiert, oder eine allelische Variante, eine Modifikation oder ein Analog dieser DNA-Sequenz ist, die (das) für ein Protein kodiert, das die biologisch aktiven Eigenschaften von nativem ECGF beibehält.

20. cDNA-Klon nach Anspruch 19, enthaltend untranslatierte Nukleotidsequenzen, die 5' und 3' von der kodierenden Sequenz des humanen Endothelzellwachstumsfaktors angeordnet sind, definiert durch die mRNA für Endothelzellwachstumsfaktor.
21. Zusammensetzung, umfassend eine therapeutisch wirksame Menge von humanem Endothelzellwachstumsfaktor, wie in Anspruch 17 oder 18 definiert, zur Förderung der Wundheilung in einer Mischung mit einem vertraglichen Träger.

Patentansprüche für folgenden Vertragsstaat : AT

1. Verfahren zum Erzeugen von humanem Endothelzellwachstumsfaktor, umfassend das Bereitstellen eines replikationsfähigen Expressionsvektors, der zur Expression der DNA-Sequenz, die für humanen Endothelzellwachstumsfaktor kodiert, in einem geeigneten Wirt fähig ist, das Transformieren des Wirtes zum Erhalten eines rekombinanten Wirtes und das Halten des rekombinanten Wirtes unter Bedingungen, die die Expression der für den Endothelzellwachstumsfaktor kodierenden cDNA-Sequenz erlauben, um Endothelzellwachstumsfaktor zu erzeugen, wobei der humane Endothelzellwachstumsfaktor die Sequenz von α -ECGF oder β -ECGF hat, die in Figur 8 gezeigt ist, oder eine allelische Variante, eine Modifikation oder ein Analog des α -ECGFs oder β -ECGFs ist, das die biologisch aktiven Eigenschaften von nativem ECGF beibehält.
2. Verfahren nach Anspruch 1 einschließlich des weiteren Schritts der Wiedergewinnung des Endothelzellwachstumsfaktors.
3. Verfahren nach Anspruch 2, worin der Expressionsvektor ein Bakteriophage ist.
4. Verfahren nach Anspruch 3, worin der Bakteriophage ein Mitglied der aus Lambda gt₁₀ und Lambda gt₁₁ bestehenden Gruppe ist.
5. Verfahren nach Anspruch 2, worin der Expressionsvektor ein Plasmid ist.

6. Verfahren nach Anspruch 5, worin das Plasmid von pBR322 abgeleitet ist.
7. Verfahren nach Anspruch 2, worin die Kontrollfunktion auf dem Expressionsvektor durch virales Material bereitgestellt wird.
8. Verfahren nach Anspruch 7, worin das virale Material von einem Mitglied der aus Rinderpapillomavirus, Epstein-Barr-Virus, Adenovirus, Affenvirus 40 (SV40) und Bakulovirus bestehenden Gruppe abgeleitet ist.
9. Verfahren zum Herstellen eines replikationsfähigen Expressionsvektors, der Endothelzellwachstumsfaktor, erhältlich nach einem der Ansprüche 1 bis 8, in einem selbstreplizierenden rekombinanten System exprimieren kann, wobei ein DNA-Insert, das für ECGF, wie erhältlich nach einem der Ansprüche 1 bis 8, kodiert, durch rekombinante DNA-Technologie in einen replizierbaren Expressionsvektor eingeführt wird.
10. Verfahren zum Herstellen eines selbstreplizierenden rekombinanten Systems, das Endothelzellwachstumsfaktor, wie erhältlich nach einem der Ansprüche 1 bis 8, exprimiert, wobei ein selbstreplizierendes System mit einem gemäß Anspruch 9 erhältlichen Vektor transformiert wird.
11. Verfahren nach Anspruch 10, worin das System in einer Zelle ist.
12. Verfahren nach Anspruch 10, worin das System zellfrei ist.
13. Verfahren nach Anspruch 10, worin ein Mitglied der aus *E. coli*, *B. subtilis*, Insekten, Hefe oder Vertebratenzellen bestehenden Gruppe transformiert oder infiziert wird.
14. Verfahren nach Anspruch 10, worin ein eukaryontischer Organismus transformiert wird.
15. Verfahren nach Anspruch 1, worin das Wiedergewinnen des Endothelzellwachstumsfaktors die Reaktion der von dem rekombinanten Wirtssystem exprimierten Proteine mit einer Reagenzzusammensetzung umfaßt, die mindestens ein Bindeprotein umfaßt, das für Endothelzellwachstumsfaktor spezifisch ist.
16. Verfahren nach Anspruch 2, worin der humane Endothelzellwachstumsfaktor im wesentlichen frei von anderen Proteinen menschlichen Ursprungs ist.



17. Verfahren nach Anspruch 16, worin der Endothelzellwachstumsfaktor eine Polypeptidsequenz umfaßt, die sich von der NH₂-terminalen Aminosäure des Endothelzellwachstumsfaktors erstreckt.

18. Verfahren zum Herstellen eines cDNA-Klones, umfassend die vollständige kodierende Sequenz für humanen Endothelzellwachstumsfaktor, wobei die cDNA eine DNA-Sequenz hat, die für α -ECGF oder β -ECGF, gezeigt in Figur 8, kodiert, oder eine allelische Variante, eine Modifikation oder ein Analog der DNA-Sequenz ist, die (das) für ein Protein kodiert, das die biologisch aktiven Eigenschaften von nativem ECGF beibehält, wobei der cDNA-Klon auf an sich bekannte Weise hergestellt wird.

19. Verfahren nach Anspruch 18, wobei der cDNA-Klon nicht translatierte Nukleotidsequenzen, die 5' und 3' von der kodierenden Sequenz des humanen Endothelzellwachstumsfaktors angeordnet sind, definiert durch die Endothelzellwachstumsfaktor-mRNA, enthält.

20. Verfahren zum Herstellen einer Zusammensetzung zur Förderung der Wundheilung, umfassend das Vereinen einer therapeutisch wirksamen Menge von humanem Endothelzellwachstumsfaktor, wie erhältlich nach einem der Ansprüche 1 bis 8, und eines akzeptablen Trägers.

Revendications

Revendications pour les Etats contractants suivants : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. Procédé de production de facteur de croissance de cellule endothéliale humaine selon lequel on met à la disposition un vecteur d'expression répliquable capable d'exprimer la séquence d'ADN codant pour le facteur de croissance de cellule endothéliale humaine dans un hôte approprié, on transforme cet hôte pour obtenir un hôte recombinant et on maintient cet hôte recombinant dans des conditions permettant l'expression de la séquence d'ADN codant pour ce facteur de croissance de cellule endothéliale, afin de produire le facteur de croissance de cellule endothéliale, ce facteur de croissance de cellule endothéliale comportant la séquence de l'ECGF- α ou de l'ECGF- β illustrée sur la figure 8, ou consistant en un variant allélique, un produit de modification ou un analogue de cet ECGF- α ou ECGF- β , conservant les propriétés biologiquement actives de l'ECGF natif.

2. Procédé selon la revendication 1, comprenant en outre l'opération de récupération de ce facteur de croissance de cellule endothéliale.

5 3. Procédé selon la revendication 2, dans lequel le vecteur d'expression est un bactériophage.

10 4. Procédé selon la revendication 3, dans lequel le bactériophage est un membre du groupe constitué par λ gt₁₀ et λ gt₁₁.

5. Procédé selon la revendication 2, dans lequel le vecteur d'expression est un plasmide.

15 6. Procédé selon la revendication 5, dans lequel le plasmide est dérivé de pBR322.

20 7. Procédé selon la revendication 2, dans lequel la fonction de contrôle de ce vecteur d'expression est procurée par de la matière virale.

25 8. Procédé selon la revendication 7, dans lequel cette matière virale est dérivée d'un membre du groupe comprenant le virus du papillome bovin, le Virus d'Epstein-Barr, un adénovirus, le virus simien 40 et le baculovirus.

30 9. Facteur de croissance de cellule endothéliale qui peut être obtenu selon le procédé de la revendication 2.

35 10. Vecteur d'expression répliquable, capable d'exprimer le facteur de croissance de cellule endothéliale selon la revendication 9, dans un système recombinant auto-répliquable.

11. Système recombinant auto-répliquable transformé avec le vecteur de la revendication 10.

40 12. Système recombinant selon la revendication 11, dans lequel ce système est dans une cellule.

45 13. Système recombinant selon la revendication 11, dans lequel ce système est sans cellule.

50 14. Système recombinant selon la revendication 11, obtenu par transformation ou infection d'un membre d'un groupe comprenant E. coli, B. subtilis, un insecte, une levure et une cellule de vertébré.

55 15. Système recombinant selon la revendication 11, obtenu par transformation d'eucaryotes.

16. Procédé selon la revendication 1, dans lequel la récupération de ce facteur de croissance de cellule endothéliale, comprend la réaction des



protéines exprimées par le système-hôte recombinant, avec une composition à base de réactif comprenant au moins une protéine de liaison spécifique du facteur de croissance de cellule endothéliale.

17. Facteur de croissance de cellule endothéliale humaine, ayant la séquence de l'ECGF- α ou de l'ECGF- β illustrée sur la figure 8, ou consistant en un variant allélique, un produit de modification ou un analogue de cet ECGF- α ou ECGF- β , conservant les propriétés biologiquement actives de l'ECGF natif, ce facteur de croissance de cellule endothéliale humaine étant à peu près exempt d'autres protéines d'origine humaine.

18. Facteur de croissance de cellule endothéliale selon la revendication 17, comprenant une séquence polypeptidique s'étendant à partir de l'acide aminé NH₂-terminal du facteur de croissance de cellule endothéliale.

19. Clone d'ADNc comprenant la séquence complète codant pour le facteur de croissance de cellule endothéliale, cet ADNc ayant la séquence d'ADN codant pour l'ECGF- α ou l'ECGF- β illustrée sur la figure 8, ou consistant en un variant allélique, un produit de modification ou un analogue de cette séquence d'ADN codant pour une protéine conservant les propriétés biologiquement actives de l'ECGF natif.

20. Clone d'ADNc selon la revendication 19, contenant des séquences nucléotidiques non traduites situées aux extrémités 5' et 3' de la séquence codant pour le facteur de croissance de cellule endothéliale humaine, ainsi que cela est défini par l'ARNm de facteur de croissance de cellule endothéliale.

21. Composition comprenant une quantité thérapeutiquement efficace de facteur de croissance de cellule endothéliale humaine ainsi que cela est défini dans la revendication 17 ou 18 afin de favoriser la cicatrisation d'une plaie, en mélange avec un véhicule acceptable.

Revendications pour l'Etat contractant suivant : AT

1. Procédé de production de facteur de croissance de cellule endothéliale humaine selon lequel on met à la disposition un vecteur d'expression répliquable capable d'exprimer la séquence d'ADN codant pour le facteur de croissance de cellule endothéliale humaine dans un hôte approprié, on transforme cet hôte pour

obtenir un hôte recombinant et on maintient cet hôte recombinant dans des conditions permettant l'expression de la séquence d'ADNc codant pour ce facteur de croissance de cellule endothéliale, afin de produire le facteur de croissance de cellule endothéliale, ce facteur de croissance de cellule endothéliale comportant la séquence de l'ECGF- α ou de l'ECGF- β illustrée sur la figure 8, ou consistant en un variant allélique, un produit de modification ou un analogue de cet ECGF- α ou ECGF- β , conservant les propriétés biologiquement actives de l'ECGF natif.

2. Procédé selon la revendication 1, comprenant en outre l'opération de récupération de ce facteur de croissance de cellule endothéliale.

3. Procédé selon la revendication 2, dans lequel le vecteur d'expression est un bactériophage.

4. Procédé selon la revendication 3, dans lequel le bactériophage est un membre du groupe constitué par lambda gt₁₀ et lambda gt₁₁.

5. Procédé selon la revendication 2, dans lequel le vecteur d'expression est un plasmide.

6. Procédé selon la revendication 5, dans lequel le plasmide est dérivé de pBR322.

7. Procédé selon la revendication 2, dans lequel la fonction de contrôle de ce vecteur d'expression est procurée par de la matière virale.

8. Procédé selon la revendication 7, dans lequel cette matière virale est dérivée d'un membre du groupe comprenant le virus du papillome bovin, le virus d'Epstein-Barr, un adénovirus, le virus simien 40 et le baculovirus.

9. Procédé de préparation d'un vecteur d'expression répliquable, capable d'exprimer le facteur de croissance de cellule endothéliale tel qu'il peut être obtenu selon l'une quelconque des revendications 1 à 8, dans un système recombinant auto-répliquable, dans lequel on introduit un segment d'insertion d'ADN codant pour le facteur ECGF qui peut être obtenu selon l'une quelconque des revendications 1 à 8, dans un vecteur d'expression répliquable en mettant en oeuvre la technologie de l'ADN recombinant.

10. Procédé de préparation d'un système recombinant auto-répliquable, exprimant un facteur de croissance de cellule endothéliale, tel qu'il peut être obtenu selon l'une quelconque des revendications 1 à 8, dans lequel on transfor-



me un système autorépliquable à l'aide d'un vecteur qui peut être obtenu selon la revendication 9.

11. Procédé selon la revendication 10, dans lequel le système est une cellule.

12. Procédé selon la revendication 10, dans lequel le système est exempt de cellule.

13. Procédé selon la revendication 10, dans lequel on transforme ou on infecte un membre du groupe consistant en *E. coli*, *B. subtilis*, un insecte, une levure et une cellule de vertébré.

14. Procédé selon la revendication 10, dans lequel on transforme un organisme eucaryote.

15. Procédé selon la revendication 1, dans lequel la récupération de ce facteur de croissance de cellule endothéliale, comprend la réaction des protéines exprimées par le système-hôte recombinant, avec une composition à base de réactif comprenant au moins une protéine de liaison spécifique du facteur de croissance de cellule endothéliale.

16. Procédé selon la revendication 2, dans lequel le facteur de croissance de cellule endothéliale humaine, est à peu près exempt d'autres protéines d'origine humaine.

17. Procédé selon la revendication 16, dans lequel le facteur de croissance de cellule endothéliale, comprend une séquence polypeptidique s'étendant à partir de l'acide aminé NH₂ terminal du facteur de croissance de cellule endothéliale.

18. Procédé de préparation d'un clone d'ADNc comprenant la séquence complète codant pour le facteur de croissance de cellule endothéliale humaine, cet ADNc ayant la séquence d'ADN codant pour l'ECGF- α ou l'ECGF- β illustrée sur la figure 8, ou consistant en un variant allélique, un produit de modification ou un analogue de cette séquence d'ADN codant pour une protéine conservant les propriétés biologiquement actives de l'ECGF natif, selon lequel on prépare ce clone d'ADNc de façon connue en soi.

19. Procédé selon la revendication 18, dans lequel le clone d'ADNc contient des séquences nucléotidiques non traduites situées aux extrémités 5' et 3' de la séquence codant pour le facteur de croissance de cellule endothéliale humaine, ainsi que cela est défini par l'ARNm

de facteur de croissance de cellule endothéliale.

20. Procédé de préparation d'une composition favorisant la cicatrisation d'une plaie, selon lequel on associe une quantité thérapeutiquement efficace de facteur de croissance de cellule endothéliale humaine tel qu'il peut être obtenu selon l'une quelconque des revendications 1 à 8, et un véhicule acceptable.