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<td>(74) Agent:</td>
<td>FASSE, J., Peter; Fish &amp; Richardson, P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).</td>
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<tr>
<td>(54) Title:</td>
<td>TREATMENT OF CENTRAL NERVOUS SYSTEM ISCHEMIA OR TRAUMA WITH EPIDERMAL GROWTH FACTOR–LIKE POLYPEPTIDES</td>
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<tr>
<td>(57) Abstract</td>
<td>The present invention features methods for preventing, reducing, or eliminating a neurological deficit caused by an injury to the central nervous system (CNS). The methods can be carried out, for example, by administering a polypeptide in the epidermal growth factor (EGF) family to a patient who has such a deficit.</td>
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TREATMENT OF CENTRAL NERVOUS SYSTEM ISCHEMIA OR TRAUMA
WITH EPIDERMAL GROWTH FACTOR-LIKE POLYPEPTIDES

Cross-Reference to Related Application

This application claims the benefit of U.S. Serial No. 60/095,830, filed August 7, 1998, which is incorporated herein by reference.

Field of the Invention

The invention relates to the treatment of neurological deficits caused by injuries to the central nervous system.

Background of the Invention

Neurotrophic factors are polypeptides that are required for proper development of the nervous system. The first neurotrophic factor discovered, nerve growth factor (NGF), is now known to be a part of a large family of growth factors, as is epidermal growth factor (EGF), which was discovered a short time later. The EGF family includes EGF, transforming growth factor-α (TGF-α), vaccinia growth factor (VGF), amphiregulin (AR), heparin binding-EGF (HB-EGF), betacellulin (BTC), epiregulin, neuregulin-1 (NRG-1, which is also known as Neu differentiation factor (NDF), heregulin (HRG), acetylcholine receptor-inducing activity (ARIA) and glial growth factor (GGF)), and neuregulin-2 (NRG-2). These polypeptides contain a common amino acid motif, the EGF-domain, which consists of about 40-45 amino acid residues, including six highly conserved cysteine residues that are arranged as follows: $\text{CX}_n\text{CX}_4\text{CX}_{10}\text{CX}_n\text{CX}_4\text{C}$ (SEQ ID NO:3; where C is cysteine and $X_i$ is any seven amino acids, $X_i$ is any four amino acids, and so on). The six cysteine residues form three intramolecular disulfide bonds (between the first and third, the second and
fourth, and the fifth and sixth cysteine residues of SEQ ID NO:3), which are essential for HB-EGF mitogenic activity.

Another common feature of polypeptides in the EGF family is that they are synthesized as transmembrane precursor molecules and then proteolytically processed so that the mature growth factor is released from the cell surface in a soluble form.

While the ability of polypeptide growth factors to support cell growth and differentiation in the developing animal has become increasingly evident, their role in the mature animal, particularly in the nervous system, is much less clear. This is unfortunate because, when neurons are injured or die in a mature animal, permanent motor and cognitive deficits can result. Even when there is some recovery over time, patients who suffer any form of cerebral ischemic episode often remain mildly to severely debilitated.

Summary of the Invention

The present invention features methods for preventing, reducing, or eliminating a neurological deficit (i.e., a cognitive or sensorimotor deficit) caused by an injury to the central nervous system (CNS). The methods can be carried out by administering a polypeptide in the epidermal growth factor (EGF) family to a patient who has or is at risk of incurring such a deficit. Polypeptides in the EGF family are referred to herein (and are defined further below) as "EGF-like polypeptides."

The present methods can be applied to reduce or eliminate a neurological deficit after an injury has occurred (i.e., after an injury that causes or precipitates the deficit). This is advantageous because there is often no way to anticipate when such an injury


will occur. For example, traumatic injuries, such as those caused by automobile accidents or during sporting events, as well as all forms of stroke, are unpredictable. Surprisingly, EGF-like polypeptides can reduce or eliminate a sensorimotor or cognitive deficit even when administration commences long after an injury has occurred. For example, administration can begin more than 6, more than 12, more than 24, more than 48, or more than 72 hours following an injury, and still provide a significant benefit. Indeed, administration may beneficially commence weeks, months, or years after an injury. Of course, administration can also begin immediately after an injury. A further surprising advantage of the invention is that administration can be carried out intravenously; the EGF-like polypeptides of the invention are effective despite the blood-brain barrier.

Thus, the invention features a method for reducing a neurological deficit in a patient who has suffered an injury to the central nervous system by administering to the patient an amount of an EGF-like polypeptide effective to reduce the neurological deficit in the patient. The injury can be an ischemic episode (e.g., a focal or global ischemic episode) or a traumatic injury. The EGF-like polypeptide, as described herein, can be EGF; TGFα; VGF; AR; HB-EGF; BTC; epieregulin; a neuregulin; or an EGF receptor-binding fragment or analog thereof (e.g., the EGF domain of any of these EGF-like polypeptides). In addition to fragments, EGF-like polypeptides containing one or more conservative amino acid substitutions are within the scope of the invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and
materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

**Brief Description of the Drawings**

Fig. 1 is a representation of the nucleic acid sequence of human heparin-binding epidermal growth factor (HB-EGF) (SEQ ID NO:1) and the amino acid translation (SEQ ID NO:2).

**Detailed Description**

Neurological deficits can be caused by injuries to the brain or spinal cord. As described below, a patient who has sustained such an injury can be treated with an EGF-like polypeptide, which will prevent, reduce, or eliminate the deficits that have occurred or would be expected to occur. Suitable polypeptides, appropriate modes of administration, injuries amenable to treatment, and means of analyzing the results obtained from a treatment regimen are described below.

A. **Polypeptides**

Polypeptides useful in the context of the present invention are polypeptides in the EGF family ("EGF-like polypeptides"). Those of ordinary skill in the art are
well able to identify a polypeptide in this family, as it will have a recognizable EGF domain (the consensus sequence being Cx₅CX₆CX₁₀CX₅C (SEQ ID NO:3), as described above) or an ability to activate an EGF receptor.

Four members of the EGF receptor family have been identified and designated using the HER (Human EGF Receptor) or erbB nomenclature. These receptors are: the classical EGF receptor (EGFR), also known as HER1 or erbB1; HER2 or erbB2, also known as p185 or neu; HER3 or erbB3; and HER4 or erbB4. EGF receptors contain a single ectodomain to which polypeptides in the EGF family bind, a single membrane spanning domain, and an intrinsic kinase domain in the cytoplasm. Upon ligand binding, the monomeric receptors form homodimers or heterodimers with other members of the EGFR family, a process known as transmodulation, and phosphorylate specific tyrosine residues within the cytoplasmic domain. These phosphorylated tyrosine residues act as docking sites for effector molecules resulting in activation of signal transduction pathways. The four EGF receptor sub-types share a high degree of homology, most notably in the 290-amino acid cytoplasmic tyrosine kinase domain. Two cysteine-rich regions in the extracellular ligand-binding domain are also conserved. In contrast, very little homology exists in the 100-amino acids at the C-terminal that are essential for the activation of intracellular signal transduction pathways after ligand binding.

EGF-like polypeptides exhibit different specificities in binding and activating EGF receptors (see Table 1). EGF, transforming growth factor-alpha (TGF-α), and amphiregulin (AR) bind to HER1; neuregulin-1 (NRG-1) and neuregulin-2 (NRG-2) bind to HER3 and HER4, but only activate HER4; HB-EGF and BTC bind and activate both HER1 and HER4. No ligand has been identified so far for the receptor HER2.
For a review of EGF receptors, including specific binding and sequence information, signalling, and receptor topology, one can consult, for example, McInnes and Sykes (Biopolymers 42:339-366, 1997), Boonstra et al. (Cell Biol. Intl. 19:413-430, 1995), or Gill (Mol. Reprod. Dev. 27:46-53, 1990).

As used herein, the terms "protein" and "polypeptide" both mean any chain of amino acid residues, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). The polypeptide growth factors useful in the invention are "substantially pure," meaning that a composition containing the polypeptide is at least 60% by weight (dry weight) the polypeptide of interest, e.g., an EGF-like polypeptide such as HB-EGF. Preferably, the polypeptide-containing composition is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, the polypeptide of interest. Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Any EGF-like polypeptide that binds to and activates an EGF receptor can be used to treat (i.e., prevent, reduce, or eliminate) a sensorimotor deficit caused by CNS ischemia or trauma. As used herein, the term "bind(s) to and activate(s)" refers to a specific interaction between an EGF-like polypeptide and an EGF receptor that results in signal transduction sufficient to elicit a biological response that contributes to the prevention or reduction of a neurological deficit. An EGF-like polypeptide of the invention will bind to an EGF receptor with an affinity that is equivalent to at least 50%, more preferably at least 70%, and most preferably at least 90% (e.g., 95%, 97%, or even 99%, 100%, or more than 100%) of the binding affinity of a naturally
occurring EGF family member for its cognate receptor (ligand-receptor binding partners are indicated in Table 1) (e.g., see Twardzik et al., Proc. Natl. Acad. Sci. USA 82:5300-5304, 1985 for a comparison of the binding affinity of VGF, TGFα, and EGF for the EGF receptor).

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One of ordinary skill in the art is readily able to identify an EGF-like polypeptide. Assays for receptor binding and activation are routinely practiced in the art. In the context of the present invention, these assays can be based, for example, on the ability of both mouse and human EGF to compete with 125I-labeled mEGF for
binding sites on human foreskin fibroblasts. For example, $^{125}$I-labeled mEGF binding and competition assays can be carried out on monolayer cultures of human foreskin fibroblasts (e.g., approximately $1 \times 10^6$ cells/60 mm Falcon dish) by incubating a standard amount of $^{125}$I-labeled mEGF in the simultaneous presence of aliquots containing increasing amounts of a competing peptide (e.g., EGF-like polypeptides and substitution and deletion mutants thereof) for 1 hour at 37°C. The binding medium can consist of 1.5 ml of an albumin-containing modified Dulbecco medium. Unbound $^{125}$I-labeled mEGF can be removed by washing, and the cells can be solubilized by the addition of 1 ml of 0.5 M NaOH. Radioactivity can then be measured with a gamma spectrometer (available, e.g., from Nuclear-Chicago). One can then generate a standard curve by plotting the percentage of $^{125}$I-labeled mEGF displaced versus the amount of the polypeptide applied (Cohen and Carpenter, Proc. Natl. Acad. Sci. USA 72:1317-1321, 1975).

Similarly, a radioreceptor assay can be carried out on monolayers of A431 cells that have been fixed on 24-well plates with 10% formalin in phosphate buffered saline (PBS). Formalin-fixed cells do not slough off plates as easily as do unfixed cells, and replicate values are thus more consistent. Under these assay conditions, $^{125}$I-EGF (1 x $10^{10}$ cpm/nmol) saturates the binding assay at 3 nM; assays can be performed at 10% of the saturation value (Twardzik et al., Proc. Natl. Acad. Sci. USA 82:5300-5304, 1985).

The invention encompasses "functional EGF-like polypeptides," which possess one or more of the biological functions or activities of the polypeptide growth factors described herein (i.e., the EGF-like polypeptides known in the art and listed in Table 1). These functions or activities are those required to
elicit a reduction in a neurological deficit caused by an
injury to the CNS (i.e., those functions or activities
that reduce or eliminate a sensorimotor deficit, a speech
impediment, a visual impairment, the loss of a cognitive
skill, or the loss of one’s ability to form accurate
memories). Accordingly, alternate molecular forms of
EGF-like polypeptides are within the scope of the
invention (e.g. fragments and other mutants, and analogs
with one or more conservative amino acid substitutions
compared to wild type EGF-like polypeptides).

EGF-like polypeptides containing one or more
conservative amino acid substitutions that do not destroy
the biological activity of the EGF-like polypeptide
(particularly the ability of the EGF-like polypeptide to
prevent, reduce, or eliminate sensorimotor deficits) are
useful. Conservative amino acid substitutions include
substitutions within the following groups: glycine and
alanine; valine, isoleucine, and leucine; aspartic acid
and glutamic acid; asparagine and glutamine; serine and
threonine; lysine and arginine; and phenylalanine and
tyrosine. Thus, EGF-like polypeptides containing one or
more conservative amino acid substitutions (preferably at
positions other than the six conserved cysteine residues
in the EGF domain) are within the scope of the invention.

An EGF-like polypeptide that consists of a mutant
of one of the full length polypeptides listed in Table 1
will fall within the scope of the invention when, at a
given concentration, the EGF-like polypeptide displaces
at least 50%, preferably at least 70%, and more
preferably at least 90% (e.g., 95%, 97%, or even 99%) as
much $^{125}$I-labeled mEGF as a wild-type human EGF
polypeptide in one of the binding assays described
herein. For example, this can be determined by
performing a standard receptor binding assay, such as

A mutant EGF-like polypeptide of the invention can be a fragment of a full-length polypeptide disclosed in Table 1. For example, a mutant EGF-like polypeptide can consist of the EGF domain of an EGF-like polypeptide (i.e., the domain conforming to the consensus sequence CX_{3}CX_{4}CX_{10}CXCX_{9}C (SEQ ID NO:3), e.g., amino acid residues 108-143 of SEQ ID NO:2) or the EGF domain and one or more of the amino acid residues that flank the EGF domain. Truncated polypeptides (i.e., fragments of an EGF-like polypeptide in which one or more of the amino acid residues on one or more of the terminal ends of an EGF-like polypeptide) can be used to practice the invention. For example, a deletion of 1, 2, 5, 10, or more amino acid residues from the amino and/or carboxy terminals of EGF, TGF-α, AR, HB-EGF, BTC, epieregulin, or a neuregulin would result in a mutant EGF-like polypeptide useful in the present invention. For example, HB-EGF polypeptides useful in the invention include those that include amino acid residues 2-208; 6-208; 11-208; 100-208; 100-145; 1-207; 1-202; 1-198; and the like (all of SEQ ID NO:2). Thus, EGF-like polypeptides useful in the invention can consist of active fragments of polypeptides in the EGF family. By "active fragment" is meant any portion of an EGF-like polypeptide that, when tested in a receptor binding assay such as those described above, displaces at least 50% of the ligand that would otherwise bind to the receptor (e.g., a fragment of HB-EGF is an active fragment of HB-EGF if it displaces at least 50% as much ^{125}I-labeled mEGF as a wild-type human EGF polypeptide). Active or functional fragments of an EGF-like polypeptide can also be assessed by their ability to improve the neurological deficits (i.e., the cognitive and sensorimotor deficits) that can result following a
traumatic or ischemic injury. The ability of any given EGF-like polypeptide to improve these deficits can be assessed by tests of cognition and motor skill that are well known to those of ordinary skill in the art. Prior to clinical trials, the efficacy of an EGF-like polypeptide in improving a neurological deficit can be assessed in animal models (see the forelimb placing test, the hindlimb placing test, the modified balance beam test, and the postural reflex test described below). The active fragment will produce at least 40%, preferably at least 50%, more preferably at least 70%, and most preferably at least 90% (including 100% or more) of the benefit of the full-length polypeptide. The activity of any given polypeptide or fragment thereof can be readily determined in any number of ways. For example, a fragment of an EGF-like polypeptide that, when administered according to the methods of the invention described herein, is shown to produce performance in functional tests that is comparable to the performance that is produced by administration of the full-length EGF-like polypeptide, the fragment would be an "active fragment" of the EGF-like polypeptide and encompassed by the invention. It is well within the abilities of those of ordinary skill in the art to determine whether an EGF-like polypeptide, regardless of, e.g., size, retains the functional activity of the full length, wild type EGF-like polypeptide.

A description of particular EGF-like polypeptides follows.

1. **Heparin-binding epidermal growth factor**

Heparin-binding EGF (HB-EGF) was initially identified in the conditioned medium (CM) of macrophage-like U-937 cells (Higashiyama et al., *Science* 251:936-939, 1991). It is a growth factor with a strong affinity for immobilized heparin that is mitogenic for Balb/c 3T3
cells and smooth muscle cells (SMC) but not for endothelial cells (EC). HB-EGF has been purified from the CM of macrophage-like human U-937 cells using heparin-affinity chromatography and reverse phase liquid chromatography and cloned from a U-937 cell cDNA library. Analysis of HB-EGF cDNA revealed an open reading frame that predicts a primary translation product of 208 amino acids (SEQ ID NO:2) containing a putative signal peptide, a propeptide, the mature HB-EGF, and transmembrane and cytoplasmic domains (see, e.g., U.S. Patent No. 5,811,393). For a description of the structural organization and chromosomal assignment of human HB-EGF, see Pen et al. (Biochem. 32:7932-7938, 1993).

Various forms of HB-EGF are useful in the present invention. For example, a 66 amino acid form (amino acid residues 82-147 of SEQ ID NO:2), an 86 amino acid form (amino acid residues 63-148 of SEQ ID NO:2), the full-length, 208 amino acid form (SEQ ID NO:2), as well as any EGF receptor-binding forms of HB-EGF that have undergone post-translational modification or processing can be used to practice the invention. Such post-translational modification may include, without limitation, a processed amino-terminus, for example, removal of all or part of a signal sequence or all or part of a pro sequence; a processed carboxy-terminus, for example, removal of all or part of a membrane-spanning domain or all or part of a cytoplasmic domain; 0-linked glycosylation; or any combination thereof.

One source of HB-EGF is the human histiocytic lymphoma cell line U-937, which is available from the American Type Culture Collection (Manassas, VA) under Accession No. CRL 1593. Methods of purification are known to those of ordinary skill in the art (for particular guidance on purification of HB-EGF, see U.S. Patent No. 5,811,393). HB-EGF and other EGF-like
polypeptides of the invention can also be recombinantly
or synthetically produced. Thus, polypeptide growth
factors useful in the invention can be naturally
occurring, synthetic, or recombinant molecules and can
consist of a hybrid or chimeric polypeptide with one
portion, for example, being an EGF-like polypeptide, and
a second portion being a distinct polypeptide (e.g., a
large molecule such as albumin or a portion of an
immunoglobulin). Methods by which EGF-like polypeptides
can be purified from a biological sample, chemically
synthesized, or produced recombinantly by standard
techniques are well known (see e.g., Ausubel et al.,
Current Protocols in Molecular Biology, New York, John
Wiley and Sons, 1993; Pouwels et al., Cloning Vectors:

The HB-EGF gene is normally expressed in a variety
of tissues, including the lung, heart, and skeletal
muscle. HB-EGF has been implicated as a participant in a
variety of normal and aberrant processes such as
blastocyst implantation, wound healing, SMC hyperplasia,
tumor growth, and atherosclerosis (see, e.g., Fen et al.,
supra).

HB-EGF is also expressed in the mammalian brain.
In particular, HB-EGF mRNA is found in the cortex,
hippocampus, and other deep structures of the rat
forebrain. While neurons and oligodendrocytes appear to
normally express HB-EGF polypeptides, the studies
described below suggest that HB-EGF mRNA is upregulated
within a few days after focal brain injury (or "stroke").
The upregulation is evident in tissue surrounding focal
brain wounds or infarcts. Accordingly, it would be
reasonable to expect that HB-EGF administered prior to or
shortly after an injury to the CNS would exert a
neuroprotective effect. However, the inventors have
discovered that HB-EGF is ineffective in reducing infarct
volume following a focal ischemic injury. As described further below, an EGF-like polypeptide can be administered to a patient who has suffered an ischemic or traumatic injury to the CNS even after a substantial period of time has elapsed. Surprisingly, administration of an EGF-like polypeptide can commence more than six or twelve or more hours after the onset of the injury. In the event administration of HB-EGF (or any EGF-like polypeptide of the invention) is begun at or after this time (i.e., after neurons or glial cells in the brain are adversely affected by ischemia) the polypeptide may act by a different mechanism (i.e., a mechanism that differs from infarct volume reduction). For example, EGF-like polypeptides may reduce retrograde or secondary cell death and/or stimulate neuronal sprouting and synapse formation.

2. Betacellulin

Betacellulin (BTC) was originally purified from the conditioned media of a mouse pancreatic tumor cell line. The nucleotide sequence of BTC cDNA predicts a polypeptide of 178 amino acids, which is proteolytically processed so that a soluble 80-amino acid BTC protein is released from BTC-expressing cells. Northern blot analysis has revealed relatively high expression in lung, uterus, kidney, thymus, heart, liver, spleen, small intestine, pancreas, muscle, and testis.

3. Neuregulins

The neuregulins (heregulin, NDF, GGF, ARIA) are a large group of secreted and membrane-attached EGF-like growth factors that are expressed as alternatively spliced isoforms of a single gene. They are produced by neurons and mesenchymal cells and elicit various trophic responses such as cellular proliferation, survival, and differentiation. The secreted forms of these polypeptides are about 34-44 kDa. In general, the
neuregulins are produced by neurons and exert their effects on other types of receptor-bearing cells, such as glia and skeletal muscle cells. Mice lacking neuregulin exhibit defects in heart development and in the developing rhombencephalon, a structure that develops onto the brain stem. These animals are also devoid of Schwann cell precursors.

B. Treatment Regimens

A treatment regimen suitable for practicing the methods of the invention is any regimen that, when carried out by administering a therapeutic dose of an EGF-like polypeptide via an appropriate route and for an appropriate period of time, prevents, reduces, or eliminates a neurological deficit caused by an injury to the patient's CNS. The treatment regimen may be assessed by examining one or more of the patient's motor skills (e.g., posture, balance, grasp, or gait), cognitive skills, speech, or sensory perception (including visual ability, taste, olfaction, and proprioception), and determining whether the sensorimotor, cognitive, or other skill improves following treatment with an EGF-like polypeptide.

Administration of EGF-like polypeptides can be carried out by any known route of administration, including intravenously, orally, or intracerebrally (e.g., intraventricularly, intratheca, or intracisternally). Intracisternal administration can be carried out, e.g., using 0.1 to 100 μg/kg/injection of an EGF-like polypeptide (e.g., HB-EGF or an active fragment thereof), and administering a single injection or a series of injections. As an alternative to determining dosage based on body weight, one of skill in the art could readily administer the required dosage to cerebrospinal fluid based on the surface area of the
human brain. In this event, a human patient is typically treated with 0.01-1000 mg (e.g., 0.1, 1, 10, 100, 250, 500, 700, 800, or 900 mg) of an EGF-like polypeptide. Intracisternal administration can consist of a single application given, for example, 24 hours after an injury, a pair of applications, given, for example, 24 and 48 hours after an injury, or, if necessary, a series of applications of, for example, 1.0 mg, given biweekly (for example, every 3-4 days) in a treatment regimen that commences 24 hours or longer following the ischemic episode. The treatment regimen may last a number of weeks. Alternatively, intracisternal administration can consist of a series of applications, at, for example, 1.0 mg, given once, twice, or, for example, biweekly, in a treatment regimen that begins six, twelve, or twenty-four hours or longer following the ischemic episode.

Alternatively, the polypeptide growth factors can be administered intravenously. Typically, the dosage for intravenous administration will be greater than that for intracisternal administration, e.g., 1 to 1,000 μg/kg of an EGF-like polypeptide can be administered. Preferably, an EGF-like polypeptide is administered intravenously at concentrations ranging from 1 to 100 μg/kg/hour.

More specifically, EGF-like polypeptides can be administered to a patient at therapeutically effective doses (i.e., doses sufficient to result in functional recovery, beyond that which would be expected without administration of the polypeptide) that are determined as follows.

1. **Effective Dose**

Toxicity and therapeutic efficacy of any given EGF-like polypeptide can be determined by standard pharmaceutical procedures, using either cells in culture, experimental animals, or both, to determine the LD₅₀ (the dose that proves lethal to 50% of the population) and the
ED$_{50}$ (the dose that proves therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which is expressed as the ratio LD$_{50}$:ED$_{50}$. EGF-like polypeptides that exhibit large therapeutic indices are preferred. However, EGF-like polypeptides that exhibit toxic side effects can still be used, so long as the system for delivery targets such polypeptides to the site of affected tissue in a limited manner (i.e., a manner in which potential damage to unaffected cells is minimized and adverse side effects are thereby reduced to an acceptable level).

The data obtained from cell culture assays and animal studies, notably the studies of rats described below, can be used in formulating a range of dosage for use in humans. The dosage of such polypeptides lies preferably within a range of circulating concentrations that include the ED$_{50}$ with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration. For any EGF-like polypeptide used in a method of the invention, the therapeutically effective dose can be estimated initially from the studies of surgically induced ischemia in the mammalian brain that are described below.

A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC$_{50}$ (that is, the concentration of the test polypeptide that achieves a half-maximal induction of recovery) as determined in the in vivo studies described below. Such information can be used, if necessary, to more accurately determine therapeutically effective doses in humans. Moreover, it is well known in the medical arts that dosages for any one patient depend on many factors, including the general health, sex, weight,
body surface area, and age of the patient, as well as the particular compound to be administered, the time and route of administration, and other drugs being administered concurrently. Determining the most appropriate dosage and route of administration is well within the abilities of a physician of ordinary skill in the art.

2. Formulations and Use

Pharmaceutical compositions for use in accordance with the present invention (i.e. compositions that include an EGF-like polypeptide) can be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients, many of which are known to those of ordinary skill in the art. Excipients can be used, for example, when pharmaceutical compositions of the invention are administered intracerebrally or intravenously. It is known that another polypeptide growth factor, bFGF, can cross the blood brain barrier when administered intravenously and enters ischemic brain tissue (Fisher et al., J. Cereb. Blood Flow Metab. 15:953-959, 1995; Huang et al., Amer. J. Physiol. in press). Suitable excipients include buffers (e.g., citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (e.g., serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol.

In addition to the preferred routes of administration described above, an EGF-like polypeptide can be administered orally, intraarterially, subcutaneously, intramuscularly, intraventricularly, intracapsularly, intra-spinally, intracisternally, or transmucosally. Thus, an EGF-like polypeptide can be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or
for oral, buccal, intranasal, intravaginal, intraocular, parenteral, or rectal administration.

An EGF-like polypeptide can be formulated in various ways, depending on the route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application. For oral administration, pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. Methods for making such formulations are well known and can be found in, for
example, "Remington's Pharmaceutical Sciences"

An EGF-like polypeptide can be formulated for administration so that a given dosage can be presented either all at once or gradually over time, for example, by a continuous infusion or from an implantable slow-release device. Accordingly, formulations for injection can be presented in unit dosage form, for example, in ampules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use. When gradual release is desired, an EGF-like polypeptide can be formulated as a depot preparation. Such long acting formulations can be administered by implantation (e.g., subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (e.g., as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The EGF-like polypeptides can, if desired, be presented in a pack or dispenser device, which can contain one or more unit dosage forms containing the active ingredient. The pack can, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration.
C. Conditions Amenable to Treatment

The methods of the invention can be used to treat the adverse consequences of central nervous system injuries (i.e., a sensorimotor or cognitive deficit) that result from any of a variety of conditions, so long as those conditions are associated with an ischemic episode. By "ischemic episode" is meant any circumstance that results in a deficient supply of blood to a tissue. Cerebral ischemic episodes result from a deficiency in the blood supply to the brain. The spinal cord, which is also a part of the central nervous system, is similarly susceptible to ischemia resulting from diminished blood flow. An ischemic episode may be caused by a constriction or obstruction of a blood vessel, as occurs in the case of a thrombus or embolus. Alternatively, the ischemic episode can result from any form of compromised cardiac function, including cardiac arrest, as described above. It is expected that the invention will also be useful for treating injuries to the central nervous system that are caused by mechanical forces, such as a blow to the head or spine. Trauma can involve a tissue insult such as an abrasion, incision, contusion, puncture, compression, and the like, such as can arise from traumatic contact of a foreign object with any locus of or appurtenant to the head, neck, or vertebral column. Other forms of traumatic injury can arise from constriction or compression of CNS tissue by an inappropriate accumulation of fluid (e.g., a blockade or dysfunction of normal cerebrospinal fluid or vitreous humor fluid production, turnover, or volume regulation, or a subdural or intracranial hematoma or edema). Similarly, traumatic constriction or compression can arise from the presence of a mass of abnormal tissue, such as a metastatic or primary tumor.
Other injuries may be caused by hypertension, hypertensive cerebral vascular disease, rupture of an aneurysm, an angioma, blood dyscrasias, cardiac failure, cardiac arrest, cardiogenic shock, septic shock, head trauma, spinal cord trauma, seizure, bleeding from a tumor, or other blood loss.

Where the ischemia is associated with a stroke, it can be either global or focal ischemia. By "focal ischemia," as used herein in reference to the central nervous system, is meant the condition that results from the blockage of a single artery that supplies blood to the brain or spinal cord, resulting in the death of all cellular elements (pan-necrosis) in the territory supplied by that artery. By "global ischemia," as used herein in reference to the central nervous system, is meant the condition that results from a general diminution of blood flow to the entire brain, forebrain, or spinal cord, which causes the death of neurons in selectively vulnerable regions throughout these tissues.

The pathology in each of these cases is quite different, as are the clinical correlates. Models of focal ischemia apply to patients with focal cerebral infarction, while models of global ischemia are analogous to cardiac arrest, and other causes of systemic hypotension.

D. Experimental Models

1. Occlusion of the Middle Cerebral Artery

Occlusion of the middle cerebral artery (MCA) is a well accepted model of a focal ischemic episode and is thought to mimic the events that occur in humans following a stroke (Kawamata et al., J. Cereb. Blood Flow Metab., 16:542-547, 1996; Gotti et al., Brain Res. 522:290-307, 1990). To generate this model in rodents, one can use, for example, male Sprague-Dawley rats weighing 250-300 grams. For surgical procedures, the
animals are anesthetized with 2% halothane in
70% NO<sub>2</sub>/30% O<sub>2</sub>, and the tail artery is cannulated to
enable blood gas and blood glucose monitoring. Body
temperature can be monitored using a rectal probe and
should be maintained at 37 ± 0.5°C, for example, by
placing the animal on a heating pad. The proximal right
middle cerebral artery (MCA) is occluded permanently
using a modification of the method of Tamura et al.
the proximal MCA is exposed transcranially without
removing the zygomatic arch or transecting the facial
nerve. The artery is then electrocoagulated using a
bipolar microcoagulator from just proximal to the
olfactory tract to the inferior cerebral vein, and is
then transected (Bederson et al., Stroke 17:472-476,
1986). Animals should be observed until they regain
consciousness and can then be returned to their home
cages. Cefazolin sodium (40 mg/kg, i.p.), an antibiotic,
may be administered to all animals on the day before and
just after stroke surgery in order to prevent infection.

2. Occlusion of the Common Carotid Arteries
Using the procedures described above to
anesthetize rodents, one can, in lieu of occluding the
MCA, expose and occlude the common carotid arteries
(e.g., with a sterile suture) for variable lengths of
time (typically, the occlusion is applied for two to five
minutes). This procedure mimics a global ischemic
episode.

3. Traumatic Head Injury
Those of ordinary skill in the art are aware of
and able to induce traumatic head injuries by applying a
particular (i.e., defined) force to an animal’s skull.
Similarly, the spinal cord may be injured by surgically
aspirating or otherwise severing it according to methods
known to those of ordinary skill in the art.
E. Evaluation of Neurological Skills

To accustom experimental animals to handling, which would be necessary for behavioral/functional testing, they can be handled, for example, for three days before surgery, for 10 minutes each day.

Following is a brief description of five functional/behavioral tests that can be used to assess sensorimotor and reflex function after an injury to the CNS. The full details of these tests have been described elsewhere (Bederson et al., Stroke 17:472-476, 1986; DeRyck et al., Brain Res. 572:44-60, 1992; Markgraf et al., Brain Res. 575:238-246, 1992; Alexis et al., Stroke 26:2338-2346, 1995). Those of ordinary skill in the art are well able to assess sensorimotor deficits by other tests known and used routinely in the art.

1. The Forelimb Placing Test

Briefly, the forelimb placing test is comprised of three subtests. Separate scores are obtained for each forelimb. For the visual placing subtest, the animal is held upright by the researcher and brought close to a table top. Normal placing of the limb on the table is scored as "0," delayed placing (< 2 sec) is scored as "1," and no or very delayed placing (> 2 sec) is scored as "2." Separate scores are obtained first as the animal is brought forward and then again as the animal is brought sideways to the table (maximum score per limb = 4; in each case higher numbers denote greater deficits). For the tactile placing subtest, the animal is held so that it cannot see the table top or touch it with its whiskers. The dorsal forepaw is touched lightly to the table top as the animal is first brought forward and then brought sideways to the table. Placing each time is scored as above (maximum score per limb = 4). For the proprioceptive placing subtest, the animal is brought forward only and greater pressure is applied to
the dorsal forepaw; placing is scored as above (maximum score per limb = 2). These subscores are added to give the total forelimb placing score per limb (range = 0-10).

2. The Hindlimb Placing Test

The hindlimb placing test is conducted in the same manner as the forelimb placing test but involves only tactile and proprioceptive subtests of the hindlimbs (maximal scores 4 and 2, respectively; total score range = 0-6).

3. The Modified Balance Beam Test

The modified balance beam test examines vestibulomotor reflex activity as the animal balances on a long, narrow beam (30 x 1.3 cm) for 60 seconds. Ability to balance on the beam is scored as follows:

1 = animal balances with all four paws on top of beam;
2 = animal puts paws on side of beam or wavers on beam;
3 = one or two limbs slip off beam; 4 = three limbs slip off beam; 5 = animal attempts to balance with paws on beam but falls off; 6 = animal drapes over beam, then falls off; 7 = animal falls off beam without an attempt to balance. Animals can receive training trials (e.g., three training trials) before surgery.

4. The Postural Reflex Test

The postural reflex test measures both reflex and sensorimotor function. Animals are first held by the tail suspended above the floor. Animals that reach symmetrically toward the floor with both forelimbs are scored "0." Animals showing abnormal postures (flexing of a limb, rotation of the body) are then placed on a plastic-backed sheet of paper. Those animals able to resist side-to-side movement with gentle lateral pressure are scored "1," while those unable to resist such movement are scored "2." All functional/behavioral tests are administered just before surgery (or other means of inducing injury) and then every other day from, e.g.,
day 1 to, e.g., day 31. At each session, animals should be allowed to adapt to the testing room for 30 minutes before testing is begun.

5. Morris Water Maze

Cognitive skills and memory are routinely evaluated in rodents by the Morris Water Maze, which tests an animal's ability to remember the position of a submerged platform in a pool of water.

F. Histological Analysis of the Injured CNS

1. Determining Infarct Volume

Following an injury to the CNS (e.g., 31 days after MCA occlusion), animals can be anesthetized deeply with pentobarbital and perfused transcardially with heparinized saline followed by 10% buffered formalin. Their brains are then removed, cut into three pieces, and stored in 10% buffered formalin before dehydration and embedding in paraffin. Coronal sections (5 μm) are cut on a sliding microtome, mounted onto glass slides, and stained with hematoxylin and eosin. The area of cerebral infarcts on each of seven slices (e.g., +4.7, +2.7, +0.7, -1.3, -3.3, -5.3, and -7.3 compared to bregma) can be determined using a computer-interfaced imaging system (Bioquant, R&M Biometrix, Inc., Nashville, TN). Total infarct area per slice is determined by the "indirect method" as [the area of the intact contralateral hemisphere] - [the area of the intact ipsilateral hemisphere] to correct for brain shrinkage during processing (Swanson et al., J. Cereb. Blood Flow Metab. 10:290-293, 1990). Infarct volume is then, if desired, expressed as a percentage of the intact contralateral hemispheric volume. The volumes of infarction in cortex and striatum can also be determined separately using these methods.
Ideally, the experimenter performing intracisternal injections, behavioral testing, and histological analysis is blinded to the treatments assigned until all data have been collected. Data can be expressed as means ± SD or means ± SEM and can be analyzed by repeated measures analysis of variance (ANOVA) followed by appropriate unpaired two-tailed t-tests, with the Bonferroni correction for multiple comparisons.

2. Immunostaining for Growth Associated Protein-43

Growth Associated Protein-43 (GAP-43) is a phosphoprotein component of the neuronal membrane and growth cone that is selectively upregulated during new axonal growth in both the peripheral and central nervous systems (Skene, Ann. Rev. Neurosci. 12:127-156, 1989; Aigner et al., Cell 83:269-278, 1995; Woolf et al., Neuroscience 34:465-478, 1990; Benowitz et al., Mol. Brain Res. 8:17-23, 1990). GAP-43 has been used as a reliable marker of new axonal growth during brain development, and following brain injury or ischemia (Stroemer et al., Stroke 26:2135-2144, 1995; Benowitz et al. supra; Vaudano et al., J. Neurosci. 15:3594-3611, 1995). GAP-43 immunoreactivity (IR) can be examined in animals with focal infarcts (produced by MCA occlusion as described above) that either receive or do not receive an EGF-like polypeptide.

For histological analysis, animals are killed, for example, 3, 7, or 14 days post-stroke surgery (by MCA occlusion) by transcardial perfusion fixation with normal saline followed by 2% formaldehyde, 0.01 M sodium-m-periodate, and 0.075 M L-lysine monohydrochloride in 0.1 M sodium phosphate buffer (pH 7.4; PLP solution). Their brains are removed, post-fixed, and cut into 40 μm sections on a vibratome. The sections are cryoprotected.
Free-floating sections are successively incubated in 20% normal goat serum, a mouse monoclonal antibody to GAP-43 (1:500, clone 91E12, Boehringer-Mannheim, Indianapolis, IN), and biotinylated horse anti-mouse IgG adsorbed against rat IgG (45 μl/10 ml; Vector, Burlingame, CA). Sections are then mounted onto glass slides, air dried, immersed in gradient ethanol, and coverslipped. Brain sections from all animals at each time point (i.e., animals sacrificed 3, 7, or 14 days post-stroke surgery) were immunostained simultaneously. Control sections were processed without primary antibody and showed no specific staining.

Following immunostaining, two standard coronal sections through the cerebral infarcts can be examined; e.g., an "anterior" section at +0.2 mm compared to bregma and a "posterior" section at -2.8 mm compared to bregma. The relative changes in the intensity and extent of GAP-43 immunoreactivity (IR) can be quantified using, for example, a computer-interfaced imagining system (Bioquant, Nashville, TN) by two different methods. Adjacent brain sections, stained with hemotoxylin and eosin by standard procedures, can be used to identify the extent of the infarct. The optical density (O.D.) of a region of reliably low GAP-43 IR (the corpus callosum) may be considered the "background" value for each section.

Measurements can be made in two ways. In one way, all brain regions showing an O.D. of at least 1.5 times the O.D. of the background are identified and highlighted. The area (in mm²) of highlighted regions in the dorsolateral sensorimotor cortex is determined for each slice, and averaged among animals in each group. In a second way, specific regions of dorsolateral sensorimotor cortex are identified using a published standard rat brain atlas (Paxinos and Watson, "The Rat
Brain in Stereotaxic Coordinates," Academic Press, San Diego, CA). On "anterior" brain sections, these include the medial peri-infarct cortex (≤1 mm from the infarct border) in the ipsilateral hemisphere, and frontal cortex areas 1 and 2 (FR 1,2) and forelimb area of cortex (FL) regions in both hemispheres. On "posterior" sections, these include the medial peri-infarct region in the ipsilateral hemisphere, as well as FR 1,2 and hindlimb area of cortex (HL) regions bilaterally. The O.D. is determined for each region on each section and normalized to background. For each method, data in sham or vehicle-treated and data in sham or bFGF-treated animals can also be determined. Data in all groups are expressed as ratios compared to stroke/vehicle-treated animals.

Examples

Example 1: HB-EGF mRNA Expression

It is known that messenger RNA (mRNA) coding for HB-EGF is expressed in the central nervous system (CNS). In the present study, expression of HB-EGF mRNA and protein was examined by Northern analysis, in situ hybridization, and immunohistochemistry. Northern analysis revealed transcripts for HB-EGF in all regions of normal rat brain. In situ hybridization studies showed that neurons in various regions, including cortex, hippocampus, and deep structures, express HB-EGF mRNA. Positively labeled cells were also present in white matter, suggesting that both neurons and glia express HB-EGF mRNA.

Immunohistochemical studies with an antibody specific to proHB-EGF, a transmembrane form of HB-EGF, demonstrated ubiquitous immunoreactivity in neurons and glial cells in white matter. In view of the wide expression of its cognitive receptor, EGFR, in central nervous system neurons, these results suggest that HB-EGF
is an endogenous ligand for EGFR in the central nervous system and may play an important role in physiological conditions.

Example 2: Receptor Binding Studies

Routine cross-linking analysis using $^{125}$I-growth factors bound to cortical neuronal cells demonstrated that: (1) EGF binds to HER1, (2) NRG binds to HER4, and (3) HB-EGF and BTC bind to both HER1 and HER4.

Example 3: HB-EGF treatment of serum-starved aortic smooth muscle cells can induce basic fibroblast growth factor (bFGF) gene expression

HB-EGF is a vascular smooth muscle cell (SMC) mitogen and chemotactic factor that is expressed by endothelial cells, SMCs, monocytes/macrophages, and T lymphocytes. Both the membrane-anchored HB-EGF precursor and the secreted mature HB-EGF protein are biologically active; thus, HB-EGF may stimulate SMC growth via autocrine, paracrine, and juxtacrine mechanisms.

HB-EGF treatment of serum-starved aortic SMCs induced fibroblast growth factor (FGF)-2 (basic FGF) gene expression, but not FGF-1 (acidic FGF) gene expression. Increased FGF-2 mRNA expression was first detectable at 1 hour after HB-EGF addition, and maximal FGF-2 mRNA levels, corresponding to an approximately 46-fold increase in expression, were present after 4 hours. The effect of HB-EGF on FGF-2 mRNA levels appears to be mediated primarily by a transcriptional mechanism and requires proteins synthesized de novo. Western blot analyses indicated that HB-EGF-treated SMCs also produced an increased amount of bFGF protein.

HG-EGF induction of FGF-2 mRNA levels can be inhibited by treating cells with the anti-inflammatory glucocorticoid dexamethasone or the glycosaminoglycan heparin. Taken together, these results indicate that HB-
EGF expressed at sites of vascular injury or inflammation in vivo may upregulate FGF-2 production by SMCs.

**Other Embodiments**

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.
What is claimed is:

1. A method for reducing a neurological deficit in a patient who has suffered an injury to the central nervous system, the method comprising administering to the patient an amount of an epidermal growth factor-like (EGF-like) polypeptide effective to reduce a neurological deficit in the patient.

2. The method of claim 1, wherein the injury comprises an ischemic episode.

3. The method of claim 2, wherein the ischemic episode is a focal ischemic episode.

4. The method of claim 2, wherein the ischemic episode is a global ischemic episode.

5. The method of claim 1, wherein the injury comprises a traumatic injury.

6. The method of claim 1, wherein the EGF-like polypeptide is epidermal growth factor or an EGF receptor-binding fragment or analog thereof.

7. The method of claim 1, wherein the EGF-like polypeptide is transforming growth factor-alpha (TGFα), vaccinia growth factor (VGF), amphiregulin (AR), betacellulin (BTC), epiregulin, or a neuregulin.

8. The method of claim 1, wherein the EGF-like polypeptide is heparin-binding EGF (HB-EGF) or an EGF receptor-binding fragment or analog thereof.
9. The method of claim 8, wherein the EGF receptor-binding fragment comprises the EGF domain of HB-EGF (SEQ ID NO:3).

10. The method of claim 8, wherein the EGF receptor-binding fragment comprises a deletion of 1, 2, 5, or 10 amino acid residues from the amino or carboxyl terminals of HB-EGF.

11. The method of claim 10, wherein the EGF receptor-binding fragment comprises amino acid residues 2-208, 6-208, 11-208, 100-208, 100-145, 1-207, 1-202, or 1-198 of SEQ ID NO:2.

12. The method of claim 10, wherein the EGF receptor-binding fragment comprises amino acid residues 82-147 of SEQ ID NO:2 or amino acid residues 63-148 of SEQ ID NO:2.

13. The method of claim 8, wherein the EGF receptor-binding fragment comprises HB-EGF (SEQ ID NO:2) with one conservative amino acid substitution.

14. The method of claim 1, wherein administration of the EGF-like polypeptide commences more than 6 hours after the injury.

15. The method of claim 14, wherein administration of the EGF-like polypeptide commences more than 12 hours after the injury.

16. The method of claim 14, wherein administration of the EGF-like polypeptide commences more than 24 hours after the injury.
17. The method of claim 1, wherein the EGF-like polypeptide is administered intravenously.

18. The method of claim 1, wherein the EGF-like polypeptide is administered intracisternally.

19. An EGF-like polypeptide for use in reducing a neurological deficit in a patient who has suffered an injury to the central nervous system.

20. The polypeptide of claim 19, wherein the EGF-like polypeptide is epidermal growth factor or an EGF receptor-binding fragment or analog thereof.

21. The polypeptide of claim 19, wherein the EGF-like polypeptide is transforming growth factor-alpha (TGFα), vaccinia growth factor (VGF), amphiregulin (AR), betacellulin (BTC), epiiregulin, or a neuregulin.

22. The polypeptide of claim 19, wherein the EGF-like polypeptide is heparin-binding EGF (HB-EGF) or an EGF receptor-binding fragment or analog thereof.

23. The polypeptide of claim 22, wherein the EGF receptor-binding fragment comprises the EGF domain of HB-EGF (SEQ ID NO:3).

Fig. 1
<110> The General Hospital Corporation

<120> TREATMENT OF CENTRAL NERVOUS SYSTEM ISCHEMIA OR TRAUMA WITH EPIDERMAL GROWTH FACTOR-LIKE POLYPEPTIDES

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)  Please See Extra Sheet.
US CL : 514/2, 12; 530/300, 350, 399; 424/85.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12; 530/300, 350, 399; 424/85.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X  Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents
*A* document defining the general state of the art which is not considered to be of particular relevance
*E* earlier document published on or after the international filing date
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"A" document member of the same patent family

Date of the actual completion of the international search  
17 NOVEMBER 1999

Date of mailing of the international search report  
9 DEC 1999

Name and mailing address of the ISA/US  
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Authorized officer  
CHRISTINE SAOUD

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*
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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):
APS, MEDLINE, EMBASE, CAPLUS
search terms: CNS, injury, EGF, heparin-binding EGF, hb-egf, ischem?, cDNA, isolat?