Title: INJECTABLE HYBRID MATRIX MIXTURES

Abstract: The invention features a method of delivering a polypeptide to an animal. The method involves introducing into the animal a fluid mixture containing: a population of cultured vertebrate cells genetically engineered to express the polypeptide; and a plurality of microcarriers.
INJECTABLE HYBRID MATRIX MIXTURES

The field of the invention is compositions used in vivo for production and delivery of medically useful substances.

Background of the Invention

5 The means used to deliver medically useful substances can significantly affect their efficacy. The standard route of administration for many such substances is either oral, intravenous, or subcutaneous. Each has inherent limitations which can affect the therapeutic utility of the substances being delivered. Furthermore, many protein-based drugs have short half-lives and low bioavailabilities, factors that must be considered in their formulation and delivery. Although various devices have been developed to deliver medically useful substances, including portable pumps and catheters, there is still a significant need for improved delivery devices.

10 Many medically useful substances, including proteins, glycoproteins, and some peptide and nonpeptide hormones, are more efficiently produced by cultured cells than via artificial synthetic routes. Appropriate cells are typically cultured in bioreactors, and the desired product purified therefrom for administration to the patient by standard means, e.g. orally or by intravenous or subcutaneous injection. Alternatively, the cells may be implanted directly into the patient, where they produce and deliver the desired product (see, e.g., U.S. application Nos. 07/787,840 and 07/789,188). While this method has a number of theoretical advantages over injection of the product itself, including the possibility that normal cellular feedback mechanisms may be harnessed to allow the delivery of physiologically appropriate levels of the product, it introduces additional complexities. One of these concerns the appropriate environment for the cells at the time of implantation. It would be desirable to organize the cells of the implant in a form that is compatible with the natural in vivo environment of the cell type comprising the implant (fibroblasts, for example, exist naturally in a rich network of extracellular matrix composed primarily of collagen). There is also a need in some cases to ensure that the implanted cells remain localized to a defined site in the patient's body, so that they can be monitored and perhaps removed when no longer needed.
U.S. Patent No. 5,965,125 is incorporated herein by reference in its entirety.

Summary of the Invention

Applicants have discovered that cells can be introduced into the body of an animal by suspending microcarriers and the cells in a fluid, and introducing the suspension into the body, e.g., by injection. The suspension can include soluble collagen in addition to the cells and microcarriers. It can also include, in suspension, solid substrates coated with a growth factor (e.g., heparin-Sepharose beads, collagen beads, collagen threads, or collagen or non-collagen fibers). These components are mixed together and delivered into the body of an animal as a liquid suspension. The delivery can be via an injectable system such as a syringe with a needle or catheter attached. The presence of the macroporous microcarriers significantly increases the surface area for cellular attachment, and can also provide protection for cells that attach and migrate into the pores of the microcarriers. The collagen portion of the mixture, if present, polymerizes to form a solid mass of collagen gel in the animal's body at the delivery site. This gel forms in situ around the suspended components (cells, microcarriers, and optional solid substrates). The gel may help to keep the injected components in a defined space, the dimensions of which will depend on the volume of the implanted material, the spatial dimensions of the implant site, and the structure of the surrounding tissue. The presence of collagen can protect the cells from the shear stress that occurs during the implantation process. In addition, collagen can help decrease the potential destruction of the implanted cells by the host's inflammatory cells by providing a protective barrier. The mixtures with collagen are called "collagen matrix mixtures" (CMM) and the solid matrices formed using the CMM are termed "collagen matrices" (CM). If desired, the microcarriers and cells can be cultured together for a period which permits the cells to adhere to the microcarriers before addition of the non-gelled collagen solution; alternatively, the three constituents can be mixed essentially simultaneously or in any desired order. When microcarriers are included, the resulting CMM is herein termed a "hybrid collagen matrix mixture" (HCMM). Mixtures without collagen or with a collagen alternative (see below) are termed "matrix mixtures"; when such mixtures contain
microcarriers, they are also designated "hybrid matrix mixture" ("HMM"). It is understood that the microcarriers can be made of substances other than, or in addition to, collagen.

The invention thus includes a mixture that contains:

(a) a population of cultured vertebrate cells (particularly mammalian cells such as cells derived from a human, chimpanzee, mouse, rat, hamster, guinea pig, rabbit, cow, horse, pig, goat, sheep, dog, or cat) that generally express a polypeptide; and

(b) a plurality of microcarriers, each of which preferably consists primarily of (i.e., greater than 50% of its dry weight is) one or more substances selected from a list including collagen (preferably type I collagen), polystyrene, dextran, polyacrylamide, cellulose, calcium alginate, latex, polysulfone, glass (e.g., glass coated with a gel such as collagen, to improve adherence of cells), and gelatin (e.g., porous gelatin or collagen). Generally at least 70%, preferably at least 80% (most preferably at least 90%, e.g., at least 95%) and up to about 100% of each microcarrier's dry weight is one or more of the listed substances. Commercial examples of microcarriers which are described as consisting essentially of purified collagen include ICN Cellagen™ Beads and HyClone Gelatin Cultispheres. The microcarriers are preferably of a porous consistency, but may be smooth, and typically have an approximately spherical shape with a diameter of approximately 0.1 to 2 mm (e.g., between approximately 0.3 and 1 mm). Of course, the shape and size of microcarriers from any particular lot or preparation will vary within manufacturing tolerances.

The cells can be derived from one or more cells removed from the patient, and preferably are genetically engineered (e.g., transfected) cells containing exogenous DNA encoding one or more polypeptides (e.g., medically useful polypeptides) such as an enzyme, hormone, cytokine, colony stimulating factor, angiogenesis factor, vaccine antigen, antibody, clotting factor, regulatory protein, transcription factor, receptor, or structural protein. Examples of such polypeptides include human growth hormone (hGH), Factor VIII, Factor IX, erythropoietin (EPO), albumin, hemoglobin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, low density lipoprotein (LDL) receptor, IL-2
receptor, globins, immunoglobulins, catalytic antibodies, the interleukins, insulin, insulin-like growth factor 1 (IGF-1), insulinotropin, parathyroid hormone (PTH), leptin, an interferon (IFN) (e.g., IFN-α, IFN-β, or IFN-γ), nerve growth factors, basic fibroblast growth factor (bFGF), acidic FGF (aFGF), epidermal growth factor (EGF), endothelial cell growth factor, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), VEGF-A, VEGF-B, VEGF-D, angiopoietins (e.g., angiopoietin 1, 2, 3, or 4), transforming growth factors, endothelial cell stimulating angiogenesis factor (ESAF), angiogenin, tissue plasminogen activator (t-PA), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), follicle stimulating hormone (FSH), α-galactosidase, β-glucosidase, α-iduronidase, α-L-iduronidase, glucosamine-N-sulfatase, α-N-acetylgalactosaminidase, acetylcoenzyme A, α-glucosaminidase-N-acetyltransferase, N-acetylgalactosamine-6-sulfatase, β-galactosidase, N-acetylglucosamine-6-sulfatase, and β-glucuronidase. Alternatively, the exogenous DNA can contain a regulatory sequence, and optionally other elements, that will activate expression of an endogenous gene (for example, using homologous recombination as described in WO94/12650-PCT/US93/11704, which is incorporated by reference herein). Suitable target endogenous genes include, for example, those encoding the proteins listed above.

Generally any type of cell which is capable of attaching to the microcarriers, and which exhibits a desirable property such as expression of a medically useful cellular product or performance of an essential structural or metabolic function, can be utilized in the mixtures of the invention. Examples include adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells and striated muscle cells, as well as precursors of any of the above. The cells are preferably cultured vertebrate cells. If desired, more than one type of cell can be included in a given mixture. The cells may be present as clonal or heterogenous populations.

While the fluid mixtures of the invention need not contain collagen, they preferably do. The collagen in the mixture is preferably type I, but may be any other
type of collagen (e.g., selected from types II, III, IV, V, VI, VII, VIII, IX, X, and XI) or any combination of two or more of such types, as well as any additional components that impart desirable characteristics to the matrix resulting from solidification of the mixture: e.g., agarose, alginate, fibronectin, laminin, hyaluronic acid, heparan sulfate, dermatan sulfate, chondroitin sulfate, sulfated proteoglycans, fibrin, elastin, tenascin, heparin or polysaccharides such as cellulose, starch, dextran or chitosan. Furthermore, either instead of collagen or in addition to collagen, the following substances can be included in the mixtures, either to endow upon matrices generated from the mixtures the properties of collagen or to enhance such properties: minced adipose tissue, minced omental tissue, methyl cellulose, alginate, gelatin, and fibrin. Any of the above mentioned collagenous and non-collagenous components may be derived from human sources or from another animal or plant source. If from an animal source and potentially immunogenic, it is preferably the same animal as the subject into which it is to be implanted. One could also include fibers (collagen or non-collagen) in the mixtures.

Collagen fibers can be in the form of cross-linked collagen threads. Non-collagen fibers can, for example, be made of a material that includes nylon, dacron, polytetrafluoroethylene, polyglycolic acid, polylactic/polyglycolic acid polymer mixtures, polystyrene, polyvinylchloride co-polymer, cotton, linen, polyester, or silk.

The microcarriers permit high concentrations of cells to be contained within the mixtures of the invention. For example, when soluble collagen is employed so that the implanted mixture forms an in situ hybrid matrix, such a hybrid matrix can contain at least approximately two (and preferably approximately three) times as many cells as a matrix prepared with a mixture containing collagen without microcarriers, assuming the number of cells inoculated and the initial production volume are equivalent. The total amount of polypeptide expressed by the cells embedded in a given hybrid matrix in a given time period is typically significantly higher (e.g., at least 25%, preferably at least 50% higher, more preferably at least 100% higher, and most preferably at least 200% higher) than achieved with a standard collagen matrix prepared from an equivalent volume of starting material.
Any of the above-described mixtures of the invention can also contain one or more (e.g., at least 2, 3, 4, 5, 6, 8, or 10) agents intended to improve the functioning matrix generated from the mixtures, e.g., by increasing proliferation and/or maintenance of the cells. These agents can include, for example, factors which promote vascularization, cytokines, or growth factors. While the agent used in a particular mixture and the polypeptide, e.g., a medically useful polypeptide, produced by the cells in the matrix can be the same substance, the two entities will generally be different. The agent can be added directly to the mixture or can be bound to or encapsulated within a solid substrate which is added to the mixture. The solid substrate can be the microcarriers themselves or can be a separate entity or entities (e.g., multiple particles of the solid substrate). The solid substrate can have heparin or heparan sulfate proteoglycan bound to it, as a means for promoting binding of the agent. An example of such a solid substrate is one that consists primarily of agarose (e.g., Sepharose™, Affi-Gel™ Heparin Gel, or Heparin-agarose), with or without heparin or heparan sulfate proteoglycan bound to it; such a solid substrate can also contain calcium alginate. Other substances from which the solid substrates can be manufactured include collagen, gelatin, ethylene-vinyl acetate, polylactide/glycolic acid co-polymer, fibrin, sucrose octasulfate, dextran, polyethylene glycol, an alginate, polyacrylamide, cellulose, latex, polyhydroxyethylmethacrylate, nylon, dacron, polytetrafluoro-ethylene, polyglycolic acid, polylactic acid, polystyrene, polyvinylchloride co-polymer, cat gut, cotton, linen, polyester, and silk. The solid substrate can be in a variety of physical forms, e.g., beads, irregular particles, sheets, or threads. When the agent is encapsulated in the solid substrate, the agent is released gradually over time. These solid substrates can function as microcarriers as well as agent reservoirs. Thus, where a particular mixture of the invention includes a solid substrate, it is not necessary that it contain any of the above-listed microcarriers.

Examples of agents which can be used in the mixtures include basic fibroblast growth factor (bFGF), VEGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, acidic fibroblast growth factor (aFGF), endothelial cell growth factor, platelet-derived growth factor (PDGF), endothelial cell stimulating angiogenesis factor (ESAF), an angiopoietin (e.g.,
angiopoietin 1, 2, 3, or 4), leukotriene C4, a prostaglandin, insulin-like growth factor 1 (IGF-1), granulocyte colony stimulating factor (G-CSF), angiogenin, transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), ascorbic acid, epidermal growth factor (EGF), and oncostatin M.

The bioactive concentration of each agent will vary greatly. A starting range is provided by the manufacturer and is usually based on a standard bioactivity assay using, for example, degree of cell proliferation as the endpoint. Typically, the agent is bound at a broad range of concentrations (lowest being what is reported as bioactive by the vendor, highest being as much as 1000x that of the reported concentration) to a substrate such as heparin-Sepharose beads; the beads are added to the mixture; a hybrid matrix is formed in vitro from the mixture; and the release of the agent over time in vitro is monitored using an appropriate detection system (e.g., an immunoassay). For these release assays, the matrices are placed in growth medium containing 10% serum. Once it is determined that the matrices release detectable amounts of the agent, a bioactivity assay is performed. Matrices containing a range of agent concentrations can, for example, be placed on porous inserts (3 to 8 μm pores) above cells that are known to proliferate in response to the agent (e.g., endothelial cells for VEGF and bFGF, fibroblasts for bFGF and PDGF, as indicated by manufacturer), and cell growth curves determined. The results of the in vitro bioactivity assay are evaluated, and doses that are not deemed bioactive as well as doses that are determined to be "toxic" (i.e., lead to cell numbers lower than control) are noted. In order to determine the optimal concentration of agent per mixture, matrices made from the mixtures containing the agent at a range of concentrations based on the in vitro bioactivity results are implanted in immunocompromised mice. Alternatively, the mixtures are introduced into the mice so that hybrid matrices are formed in situ. The optimal agent concentration per mixture is typically the concentration that allows for the maximum amount of therapeutic protein to be released for the maximum amount of time in vivo.

Instead of (or in addition to) the described agent per se being added to the mixture, the mixtures can contain, in addition to the first population of cultured vertebrate cells expressing a polypeptide (e.g., a medically useful polypeptide), a second
population of cultured vertebrate cells expressing and secreting one or more (e.g., at least 2, 3, 4, 6, 8, or 10) of the agents. The cultured vertebrate cells of the second population can be genetically engineered (as described below) to express the agent, or can be a cell which produces the agent without the benefit of genetic engineering. In the latter case, if the cell does not constitutively produce the polypeptide, or produces it in very low amounts, the cell can be induced to produce the agent, or produce it higher amounts, by gene activation. The first and second populations can be the same population of cells transfected with a DNA encoding the polypeptide and a DNA encoding the agent (or encoding the enzyme(s) necessary to produce the agent); alternatively, the cells can be transfected with a single DNA encoding both the polypeptide and the agent or agent-producing enzyme(s). The mixtures of the invention can contain additional populations of cultured vertebrate cells which express and secrete additional examples of the above described agents. The cultured vertebrate cells of the agent-producing populations can be adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, or precursors of any of the above. The cells are preferably human cells, but can be cells of any vertebrate (e.g., a mammal such as a non-human primate, pig, cow, horse, goat, sheep, dog, cat, mouse, rat, rabbit, guinea pig or hamster). When cells producing the agent are included in the mixture, one can optionally include the solid substrate as well, to bind a portion of the agent as it is secreted from the cells. This provides a means for controlling the rate of release of the agent in the mixtures or in hybrid matrices resulting from gelation or solidification of the collagen-containing mixtures.

In one preferred embodiment, the mixtures of the invention contain keratinocytes, e.g., (a) as the cells which produce the polypeptide, (b) as the cells which produce the agent, (c) both (a) and (b), or (d) as a population which produces neither the polypeptide nor the agent. Mixtures to which the keratinocytes are added are preferably those containing fibroblasts as cells producing the above-described polypeptide (e.g., a medically useful polypeptide) and/or agent. The keratinocytes and the fibroblasts can be
obtained from the same individual, and one or more keratinocyte differentiation factors (e.g., calcium ions at a concentration of 1.5-2mM, TGF-β, or keratinocyte differentiation factor-1 (KDF-1)) can be added to the mixture.

In a similar manner, the mixtures of the invention may contain endothelial cells, preferably in addition to fibroblasts producing a medically useful polypeptide, and even in addition to both fibroblasts and keratinocytes. The endothelial cells and fibroblasts can be obtained from the same or different individuals. One or more endothelial differentiation factors (e.g. vascular endothelial growth factor or basic fibroblast growth factor at 10 ng - 10 μg) can be added to the mixture to induce the formation of endothelial tubes within the hybrid matrix resulting from solidification of the collagen-containing mixture.

The mixture of the invention is generally prepared by combining, in an aqueous solution, (a) a population of any of the above-described cells; and (b) a plurality of any of the above-described microcarriers. Soluble collagen, or one or more of the alternatives listed above, is preferably also added to the mixture.

For making those mixtures containing one or more of the above-described agents, the relevant agents (free or bound to any of the above-described solid substrates) are added, together with the other components listed above, to the mixture. The agent and solid substrate can be added to the mixture together or separately, in any order.

Additionally or alternatively, a second (and, optionally, a third, fourth, fifth, sixth, or more) population of cultured vertebrate cells secreting one or more of the described agents can also be added to the mixture. Furthermore, one or more of the above-described solid substrates that include one or more substances (e.g., heparin or heparan sulfate) which binds to an agent can be added to the mixture. In addition, keratinocytes can be added to the mixture.

A polypeptide (e.g., a medically useful polypeptide) such as one listed above may be delivered to a patient by a treatment method that involves providing any of the above mixtures of the invention that contains cells (e.g., cells secreting a polypeptide of interest), and introducing the mixture into the patient in a selected site, such as a
subcutaneous, intraperitoneal, intraomental, sub-renal capsular, inguinal, intramuscular or intrathecal site. Where the polypeptide produced by the cells is one that promotes wound healing (e.g., PDGF or IGF-1), the mixture may be introduced at the site of a preexisting wound. Introduction of the mixture into a patient can be by, e.g., a pipette, a catheter or a hypodermic syringe optionally capped with a syringe needle or a catheter. The invention includes a method in which introduction of the mixture into an animal occurs at substantially the same time as forming the mixture from its components (e.g., the cultured vertebrate cells, microcarriers, and collagen). As discussed above, the cells can be derived from one or more cells removed from the patient, and are preferably transfected in vitro with exogenous DNA encoding the polypeptide. Alternatively, they may be cells which naturally secrete the polypeptide or perform the desired metabolic function (e.g., hepatocytes or pancreatic beta cells). The cells can be induced to secrete the polypeptide, secrete higher amounts of the polypeptide, or perform the desired metabolic function by gene activation. Appropriate mixtures for delivering a polypeptide to a patient can be: (a) any of those described above; or (b) any of those described above but lacking microcarriers.

The invention also includes a vessel containing any of the above-described mixtures of the invention. The vessel can be, for example, a glass or plastic hypodermic syringe, bottle, test tube, flask, or vial. It can also be a plastic bag, e.g., a blood bag. The vessel can be used, for example, for transporting a mixture of the invention from a site of manufacture or retail sale to a site of use, e.g., a hospital or a doctor's or veterinarian's office. Gelation prior to introduction of the mixture into a patient can be inhibited by, for example, maintaining the contents of the vessel at a temperature below about 8°C. Where another type of vessel is used, e.g., a vial or a bottle, the mixture can be transferred to an apparatus that is suitable for introducing the mixture into the patient (e.g., a hypodermic syringe or a blood bag) before substantial gelation has occurred. Such a vessel of the invention can be included, together with shipping material (e.g., a shipping container), in a kit.

The invention also features a kit that includes a shipping container containing (a) a first vessel containing
(i) a population of cultured vertebrate cells that express a polypeptide (e.g., cells genetically engineered to express the polypeptide), and

(ii) a plurality of microcarriers;

(b) a second vessel containing a collagen solution; and

(c) a third vessel containing a liquid medium.

The collagen solution can be at an acidic pH and the medium can be at an alkaline pH. The mixture to be introduced into an animal is prepared from the kit by: (a) combining the contents of the second and third vessels to form a collagen/medium solution; and (b) combining the collagen/medium solution with the contents of the first vessel to form the mixture. Step (b) and introduction of the liquid mixture into the subject should occur shortly after the collagen/medium solution is formed, i.e., prior to gelation of the collagen to the point the mixture is no longer a liquid. The acidic pH (in the range of about pH 2.0 to about pH 4.0) of the collagen solution in the second vessel prevents gelation of the collagen during storage. Thus, gelation of the mixture to form a solid matrix does not occur until after neutralization of the acidic collagen solution with the alkaline medium to a pH of about 7.4 to about 7.8. Alternatively, the collagen solution in the second vessel can be at a pH of about 7.4 to about 7.8, with gelation inhibited by maintaining the collagen solution at a temperature below 10°C (e.g., at a temperature of about 3°C to 8°C) until immediately prior to mixing and introduction into the animal. In either case, after formation of the mixture, it is immediately introduced into an animal, i.e., before polymerization has proceeded beyond the point the mixture can be readily transferred into the implantation site. The kit will preferably include packaging material and instructions for use in accordance with the methods of the invention.

Alternatively, the kit can include a shipping container containing two vessels with

(a) the first vessel containing

(i) a population of cultured vertebrate cells that express a polypeptide (e.g., cells genetically engineered to express the polypeptide); and
(ii) a plurality of microcarriers; and

(b) the second vessel containing a collagen solution.

The collagen solution can be at an acidic pH. In this case, the pH of the collagen solution can be adjusted to about 7.2 to about 7.4 immediately prior to formation of the mixture, using an alkaline solution, e.g., a solution of NaOH at a concentration of about 0.01N to about 1.0N. The kit can optionally include (1) a third vessel containing such an alkaline solution, and/or (2) a pH indicator such as pH paper, a dipstick, or a dye-containing solution. Alternatively, the collagen solution in the second vessel can include a pH-indicator dye.

In another embodiment, the collagen solution in the second vessel is at a pH of about 7.2 to about 7.4, with gelation inhibited by keeping the contents of the second vessel below about 8°C until immediately before formation of the mixture. Again, formation of the mixture would generally be followed immediately by introduction of the mixture into an animal, unless the mixture is maintained below 8°C to prevent gelation.

In each case, the kit will preferably include appropriate packaging material and labels and/or an insert detailing instructions for use. The packaging material can include, for example, a container suitable for shipping the mixture components to a retailer or a site of use, e.g., a hospital or a physician's office. Suitable shipping containers include, for example, plastic (or other synthetic polymer), styrofoam, cardboard, wood, or metal boxes. Paper or plastic (or other synthetic polymer) bags, envelopes, or blister packs can also be used as shipping containers. The shipping containers can also include impact protective materials such as plastic (e.g., polystyrene) fibers or chips, bubble wrap, or shredded paper, or molded plastic with slots adapted to hold the vessels, and can be thermally insulated.

The vessels used in such kits can be glass or plastic hypodermic syringes, bottles, test tubes, flasks, or vials. They can also be plastic bags, similar to blood bags. They can be supplied in a thermally insulated container in order to maintain one or
more of the components at a temperature below about 8°C prior to introduction into an animal.

In these kits, the collagen can be any of the types listed herein. Furthermore, the collagen can be substituted or supplemented with any of the alternative substances described herein. The cultured vertebrate cells can be, for example, any of those listed, and they can have been derived and/or genetically engineered by any of the procedures described herein. The polypeptides expressed by the cells and the microcarriers can be, for example, any of those listed above. If present, the noncollagen fibers indicated above can be present in any one or more of the vessels.

The kits can further contain in any of the vessels one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) agents, e.g., a factor that promotes vascularization, a cytokine, a growth factor, or ascorbic acid. The agents can be, for example, any of those listed herein. Examples of solid substrates with which the agents are optionally associated are described above. Also optionally present in a vessel of the kit is a second, third, fourth, fifth, or sixth, seventh, eighth, ninth, or tenth population of cultured vertebrate cells secreting one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) agents, e.g., a factor that promotes vascularization, a cytokine, or a growth factor, as described above.

Where the kits include fibroblasts as one of the populations of cultured vertebrate cells, a vessel of the kit can further contain endothelial cells and/or keratinocytes and, optionally, a keratinocyte differentiation factor (e.g., calcium ions at a concentration of 1.5-2 mM), as described above. The fibroblasts and the endothelial cells and/or keratinocytes can, for example, have been obtained from the same individual.

The invention also features an apparatus for introduction of a mixture into a patient. The apparatus includes at least two (e.g., two, three, four, five, or six) vessels, each of which has an outlet in fluid communication with a delivery means. The apparatus can contain (a) an aqueous collagen solution in which the collagen can be any of the collagens listed herein, (b) a population of cultured vertebrate cells that
express any of the polypeptides disclosed herein (e.g., cells genetically engineered to express any such polypeptide), and (c) a plurality of any of the microcarriers listed above. One or more of (a), (b), or (c) can be contained within each vessel. One or more of the vessels can have associated with it a piston having two ends, a first end adapted to be inserted into the interior of the vessel, and a second end adapted to extend from the interior of the vessel. The delivery means can be a syringe needle having (a) two or more inlets adapted to be in fluid communication with the outlets of the vessels, and (b) an outlet. Alternatively, the delivery means can be an adapter piece having a connector in fluid connection with the outlet of each of the vessels, a mixing chamber, and an outlet in fluid communication with a syringe needle; the connector can contain at least two inlets (e.g., two, three, four, five, or six) each in fluid communication with the outlet of only one of the vessels. The apparatus can further include one or more connections physically joining at least two vessels. In addition, the second end of each piston can be connected to a pressure plate. The apparatus can also include any of the other cell populations, alternatives to collagen, non-collagen fibers, or agents recited herein.

As used herein, a "solid substrate" is generally a plurality of objects (configured, for example, as particles or threads) that act as a reservoir or depot for a substance (e.g., a factor that promotes vascularization) contained within or bound to the solid substrate. The substance is gradually released from the solid substrate into its environment. Where the solid substrate is in the form of beads, the beads have, generally, an approximately spherical shape and a diameter of approximately 0.005 - 2.0 mm. Where the solid substrate is in the form of threads, the threads are generally about 0.01 - 1.0 mm in diameter.

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 pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although other methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents and
other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples described herein are illustrative only and not intended to be limiting.

Other features and advantages of the invention are apparent from the claims, and from the detailed description provided below.

**Brief Description of the Drawings**

Fig. 1 is a map of hGH expression plasmid pXGH302.

Fig. 2 is a graph showing the *in vivo* hGH levels in *Nude* mice implanted with either a collagen matrix or a hybrid collagen matrix containing HF165-24 cells, human skin fibroblasts stably transfected with pXGH302 and expressing hGH.

Fig. 3 is map of hFVIII expression plasmid pXF8.198.

Fig. 4 is a line graph showing levels of human factor VIII (hFVIII) in plasma from mice implanted with Heparin-Sepharose Hybrid Collagen Matrices (HSHCM) containing cells producing hFVIII and heparin-Sepharose beads either coated with basic fibroblast growth factor (bFGF) or uncoated.

Fig. 5 is a line graph showing levels of hFVIII in plasma from mice implanted with HSHCM containing cells producing hFVIII, one of two types of microcarriers, and heparin-Sepharose beads either coated with bFGF or uncoated.

Fig. 6 is a line graph showing levels of hFVIII in plasma from mice implanted with HSHCM containing cells producing hFVIII and heparin-Sepharose beads coated with various concentrations of bFGF.

Fig. 7 is a line graph showing levels of hFVIII in plasma from mice implanted with HSHCM containing cells producing hFVIII and either heparin-Sepharose beads coated with bFGF, heparin-Sepharose beads coated with vascular endothelial growth factor (VEGF), or a mixture of heparin-Sepharose beads coated with bFGF and heparin-Sepharose beads coated with VEGF.

Fig. 8 is a diagram depicting the pXVEGF.1 plasmid.
Fig. 9 is a line graph showing levels of hFVIII in plasma from mice implanted with HSHCM containing a first population of cells producing hFVIII, either alone or together with second population of cells producing VEGF.

Fig. 10 is a line graph showing the levels of hFVIII in plasma of mice injected subcutaneously with either 1mL or 2mL I-HSHCMM containing cells producing hFVIII and heparin-Sepharose beads coated with bFGF.

Fig. 11 is a map of hFVIII expression plasmid pXF8.186

Fig. 12 is a line graph showing the levels of hFVIII in plasma of mice injected intraomentally with either cells producing hFVIII, cells producing hFVIII and heparin-Sepharose beads coated with bFGF (50 ng), or cells producing hFVIII and heparin-Sepharose beads coated with VEGF (100 ng).

Fig. 13 is a line graph showing the levels of hFVIII in plasma of mice injected intraomentally with either cells producing hFVIII, cells producing hFVIII and heparin-Sepharose beads coated with bFGF (500 ng), or cells producing hFVIII and heparin-Sepharose beads coated with VEGF (100 ng).

Fig. 14 is a line graph showing the levels of hFVIII in plasma of mice injected intraomentally with cells producing hFVIII and either microcarriers, microcarriers and heparin-Sepharose beads, microcarriers and heparin-Sepharose beads coated with bFGF (500 ng), or microcarriers and heparin-Sepharose beads coated with VEGF (100 ng).

Fig. 15A is a diagram of an apparatus that can be used to simultaneously form a mixture of the invention and deliver it to a subject.

Fig. 15B is a cross-sectional view of the adapter piece 4 component of the apparatus depicted in FIG. 15A.

Detailed Description

The examples set forth below illustrate several embodiments of the invention. These examples are for illustrative purposes only, and are not meant to be limiting.
EXAMPLE I

This example describes the procedures utilized to prepare a clonal cell strain of human fibroblasts stably transfected with the plasmid pXGH302 secreting recombinant human growth hormone (hGH), and to combine them with porous collagen microcarriers in a hybrid matrix mixture of the invention. Such mixtures are referred to as hybrid collagen matrix mixtures (HCMM).

A. Generation of Primary Human Fibroblasts Expressing Human Growth Hormone

Fibroblasts were isolated from freshly excised human foreskins by an enzymatic dissociation technique. Upon confluency, primary cultures were dislodged from the plastic surface by mild trypsinization, diluted and replated to produce the secondary cell culture for transfection.

Plasmid pXGH302 was constructed as described in Example II, and transfection was carried out by electroporation, a process in which cells are suspended in a solution of plasmid DNA, placed between a pair of oppositely charged electrodes, and subjected to a brief electric pulse.

Treated cells were selected in G418-containing medium for 10-14 days. Cells that integrated the plasmid into their genomes stably expressed the product of the neo gene and formed colonies resistant to killing by the neomycin analog G418. Each colony, consisting of a clonal population of cells, was individually removed from its position on the tissue culture dish by trypsinization. Those clones scoring positive for hGH expression were expanded for quantitative assays, and clone HF165-24 was chosen for further use.

Further detailed procedures for preparing and transfecting cells suitable for use in the mixtures of this invention are provided in WO93/09222 (PCT/US92/09627), which is incorporated herein by reference.

B. Preparation of Hybrid Collagen Matrix Mixtures (HCMM)
1. Microcarrier Preparation

Collagen microcarriers (Cellnex Biosciences cat. #YB00-0015UW) were transferred from each original bottle provided by the manufacturer (~10 ml per bottle) into 50 ml conical tubes (1 tube per bottle). The microcarriers were allowed to settle in the tube, and the storage buffer solution was aspirated off. Microcarrier wash medium (DMEM with 1% calf serum and 1% penicillin/streptomycin) was added to the 50 ml mark on the graduated tube, the microcarriers allowed to settle, and the medium aspirated off. This series of washing steps was repeated for a total of 4 washes. The microcarriers were transferred to a 250 ml Erlenmeyer flask using a 25 ml plastic pipette, limiting the volume of microcarriers to 100 ml per 250 ml flask. Microcarrier wash medium was added to the 250 ml mark, and the flask was capped and placed in a tissue culture incubator at 37°C for 2-3 hours. The flask was removed from the incubator, the microcarriers allowed to settle, and the wash medium aspirated off. This series of incubation and washing steps was repeated for a total of 3 washes.

2. HCMM Preparation

The cells and microcarriers were mixed just prior to adding the collagen solution. Washed microcarriers were added to 15 ml graduated conical tube(s) to the desired volume (volume = no. of matrices multiplied by 1 ml; see Table 1). Microcarriers were allowed to settle for at least 10 minutes before measuring volume. Excess wash medium was removed by aspiration.

Cells to be included in the mixture were harvested by trypsinization and the cell number was determined. The required number of cells was centrifuged at 1500 rpm (500xg) for 7 min at room temperature. In an appropriately sized conical-bottom polypropylene tube, a mixture of equal volumes of modified 2x DMEM (2x DMEM with 9 g/L glucose, 4 mM L-glutamine, and 22.5 mM HEPES) and calf serum was prepared according to Table 1. (Note: for volumes greater than 250 ml, the total pooled volume should be divided into appropriately sized tubes.) The cell pellet was resuspended in the 2x DMEM-calf serum mixture. The collagen microcarriers were
mixed with the cell suspension by adding 1-2 ml of the cell suspension to the packed microcarriers and then transferring the concentrated microcarriers by 10 ml pipette into the remaining cell suspension, followed by gentle mixing with the pipette. The mixture was placed on ice and the appropriate volume of collagen solution was added (rat tail Type I collagen; UBI cat #08-115, diluted to concentration of 4.0 mg/ml in 0.02 M acetic acid), as indicated in Table 1. The contents of the tube were mixed carefully (avoiding creating bubbles or frothing) using a 10 ml glass pipette, until the matrix solution appeared homogenous.

To produce matrices in vitro, an appropriate volume of the collagen/cell/microcarrier/medium mix was added to a sterile petri dish with a pipette (10 or 25 ml capacity), according to the total volume per dish listed in Table 1. (The mix was agitated by pipetting occasionally during the filling of dishes to prevent settling of cells or microcarriers.) The filled dishes were placed in a 37°C, 5% CO₂, 98% relative humidity tissue culture incubator and left undisturbed for approximately 24 h, during which time the contents gelled and the size of the gel decreased in all dimensions to form hybrid matrices, which were approximately 50% of the diameter and 10% of the volume of the non-gelled mixture.
TABLE 1  Medium, Microcarrier, and Collagen Volumes Required for HCM Production

<table>
<thead>
<tr>
<th>Dish Size</th>
<th>Number</th>
<th>Modified Serum Solution</th>
<th>Microcarriers</th>
<th>Collagen Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mm</td>
<td>1</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>60 mm</td>
<td>12</td>
<td>12 ml</td>
<td>12 ml</td>
<td>12 ml</td>
</tr>
<tr>
<td>100 mm</td>
<td>1</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>100 mm</td>
<td>12</td>
<td>30 ml</td>
<td>30 ml</td>
<td>30 ml</td>
</tr>
<tr>
<td>150 mm</td>
<td>1</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>150 mm</td>
<td>12</td>
<td>90 ml</td>
<td>90 ml</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

EXAMPLE II

pXGH302 was constructed by subcloning the 6.9 kb HindIII fragment extending from positions 11,960-18,869 in the human HPRT sequence (Edwards et al., Genomics, 6:593-608, 1990; Genbank entry HUMHPRTB) and including exons 2 and 3 of the HPRT gene, into the HindIII site of pTZ18R (Pharmacia P-L Biochemicals, Inc.). The resulting clone was cleaved at the unique XhoI site in exon 3 of the HPRT gene fragment, and the 1.1 kb Sall-XhoI fragment containing the neo gene from pMC1Neo (Stratagene) was inserted, disrupting the coding sequence of exon 3. One orientation, with the direction of neo transcription opposite that of HPRT, was chosen and designated pE3neo.

To combine the hGH gene, HPRT sequences, and neo gene in the same plasmid, pXGH5 (Selden et al., Mol. Cell. Biol. 6:3173-3179, 1986) was digested with EcoRI, and the 4.0 kb fragment containing the hGH gene and linked mouse metallothionein-I (mMT-I) promoter was isolated. The EcoRI overhangs were filled in with the Klenow fragment from E. coli DNA polymerase. pE3Neo was digested with XhoI, which cuts at
the junction of the neo fragment and HPRT exon 3 (the 3' junction of the insertion into exon 3). The XhoI overhanging ends of the linearized plasmid were filled in with the Klenow fragment from \textit{E. coli} DNA polymerase, and the resulting fragment was ligated to the 4.0 kb blunt-ended mMT/hGH fragment. Bacterial colonies derived from the ligation mixture were screened by restriction enzyme analysis for a single copy insertion of the mMT-I/hGH fragment. One subclone, in which the hGH gene is transcribed in the same direction as the neo gene, was designated pXGH302. A map of plasmid pXGH302 is shown in Fig. 1. In this figure, the position and orientation of the hGH coding region and the mouse metallothionein-I promoter (mMT-I) controlling hGH expression are noted. The positions of basal promoter elements (TATA), transcription initiation sites (CAP), and translation initiation sites (ATG) are indicated. As illustrated, neo gene transcription is controlled by the polyoma enhancer/herpes simplex virus (HSV) thymidine kinase (TK) gene promoter. HPRT denotes the positions of sequences from the human hypoxanthine-guanine phosphoribosyl transferase locus. Plasmid pXGH302 utilizes the pTZ18R (Pharmacia P-L Biochemicals, Inc.) backbone, a derivative of plasmid pUC18 (Yanisch-Perron et al., \textit{Gene} 33: 103-119, 1985) carrying a T7 RNA polymerase promoter and the \textit{fl} origin of replication.

\textbf{EXAMPLE III}

This example illustrates a method of making a mixture in which transfected cells prepared as described above are precultured with the microcarriers prior to formation of the mixture. Such "precultured" hybrid collagen matrix mixtures are referred to as PCHCMM.

\textbf{A. Preculture of cells and microcarriers}

Trypsinized transfected cells are seeded onto washed collagen microcarriers at a ratio of 2 x 10^6 cells per ml microcarriers (e.g. 10 x 10^6 cells onto 5 ml microcarriers) by the following protocol:
1. Add cell suspension in a volume of growth medium that is twice the volume of the microcarriers to a 125 ml Erlenmeyer flask. The limit is 10 ml of microcarriers per flask.

2. Remove 1 - 2 ml of this suspension and add to 5 ml (packed volume) microcarriers premeasured in a 15 ml tube.

3. Transfer cell suspension/microcarriers back into the 125 ml Erlenmeyer flask.

4. Place flask into tissue culture incubator and swirl gently for approximately 5 seconds each hour for 4-5 h. Add growth medium to the 50 ml gradation on the flask, and allow cells and microcarriers to incubate undisturbed overnight.

After 20 - 24h from the time of seeding, determine the number of cells attached to collagen microcarriers by the following procedure:

1. Determine the weight of a 5 ml round bottom polystyrene test tube.

2. Remove a small sample of microcarriers (0.1 -0.2 ml) from the Erlenmeyer flask and add to the pre-weighed test tube.

3. Aspirate medium from microcarrier sample and determine the weight of the tube plus sample. Calculate sample weight by subtracting weight of tube from weight of tube plus sample.

4. Add 1 ml of matrix digestion enzyme [collagenase IA (Sigma cat #C9891) at 1 - 2 mg/ml in PBS with Ca\(^{2+}\) and Mg\(^{2+}\)] to microcarrier sample and mix gently by tapping on the side of the tube.

5. Cover the tube with parafilm and place in a 37°C water bath for 1h, promoting disintegration of microcarriers by pipetting through a Pasteur pipette at 15 minute intervals.

6. After 1h incubation, further dissociate cells by pipetting vigorously with a 5 ml glass pipette. (Note: If clumps still remain, add to the tube a solution of 10x trypsin-EDTA at a volume 1/10th that of the volume of collagenase solution added, and incubate an additional 10 min.)
7. Perform cell counts using a hemacytometer.

8. Determine density of cells per ml microcarriers using the following formula, which assumes that 50% of the wet packed volume of these microcarriers is interstitial:

\[
\text{Total number of cells/ml microcarriers} = 1000 \frac{\text{mg}}{\text{mg weight of sample}} \times (\text{cell number in sample}) \times 0.5.
\]

The cell/microcarrier mixture is transferred from the 125 ml Erlenmeyer flask to a 250 ml spinner flask (Bellco Microcarrier Spinner Flasks, 250 ml, with model #1965-60001 impeller shafts), growth medium is added to the 150 ml graduation mark, and the spinner flask is placed on a magnetic stirrer plate (set at 50 rpm) in a tissue culture incubator. The culture is fed with fresh medium the next day and 3 times weekly thereafter by allowing the microcarriers to settle on the bottom of the flask, aspirating "spent" medium to the 50 ml mark on the flask, and adding fresh growth medium to the 200 ml mark. The density of cells per ml microcarriers may be determined at desired time points as described above.

B. Preparation of PCHCMM

PCHCMM are produced by the following protocol:

1. When the desired density of cells per ml microcarriers is achieved (as determined by cell counts), remove microcarriers containing cells from the spinner flask.

2. Produce a mixture following the procedure outlined above for producing HCMM, with the following modifications:
   i. Cells are not trypsinized.
   ii. Add cultured microcarriers containing cells to 15 ml graduated conical tube(s), to the desired packed volume.
   iii. Add empty microcarriers to 15 ml graduated conical tube(s) to the desired packed volume.

3. Prepare modified 2x DMEM and calf serum mixture as described in Example I above. Add both empty microcarriers (50% of total microcarrier volume) and
microcarriers containing cells (50% of total microcarrier volume) to the modified 2x DMEM/calf serum mixture. The microcarrier/DMEM/calf serum mixture is placed on ice and the appropriate volume of collagen solution is added (e.g., rat tail Type I collagen; UBI cat #08-115, diluted to concentration of 4.0 mg/ml in 0.02M acetic acid). The contents of the tube are mixed carefully (avoiding creating bubbles or frothing) using a 10 ml glass pipette, until matrix solution appears homogenous.

Examples IV to VI and VIII to XIV contain data from experiments using various matrices formed in vitro. The results of these experiments are pertinent to the function of matrices produced by in vivo gelation following introduction of an appropriate mixture of the invention into an animal.

**EXAMPLE IV**

This example describes experiments varying the inoculum density of human fibroblasts stably transfected with the plasmid pXGH302 added to a mixture to determine the cell density that can be supported in HCM resulting from in vitro gelation of the mixture. hGH production by each HCM was also monitored.

Hybrid collagen matrices were produced in vitro with 3 inoculum densities (ID) of the stably transfected hGH-expressing neonatal foreskin fibroblast clone designated HF165-24. The densities were 5, 10, and 20 x 10^6 cells per HCM. For each ID, 9 HCM were produced in 60 mm dishes. The hybrid matrix production medium for each ID consisted of 9 ml of modified 2x DMEM, 9 ml of calf serum, 9 ml of collagen microcarriers, and 9 ml of 4 mg/ml soluble rat tail collagen, in 50 ml conical tubes.

HF165-24 harvested from monolayer cultures were pooled to provide enough of each ID for 9 HCM:

- 5 x 10^6 cells times 9 HCM = 45 x 10^6 cells total;
- 10 x 10^6 cells times 9 HCM = 90 x 10^6 cells total;
- 20 x 10^6 cells times 9 HCM = 180 x 10^6 cells total.
Pooled cells for each ID were centrifuged at 1500 rpm for 7 min, supernatant was aspirated, and pellets were resuspended in 9 ml modified 2x DMEM and 9 ml calf serum and transferred to 50 ml tubes. Nine ml of collagen microcarriers pre-measured in 15 ml graduated tubes were added to the cells/2x DMEM/calf serum mixture and mixed by gentle pipetting with a 10 ml pipette. This mixture was then placed on ice, and 9 ml of ice cold rat tail type I collagen solution (4 mg/ml) was added and mixed with a 10 ml pipette to produce a homogenous solution. Four ml of this mixture was added to each of nine 60 mm petri dishes for each density. The petri dishes were set in a tissue culture incubator and left undisturbed for 24 h. The medium was carefully aspirated from each dish after the 24 h incubation, and HCM were re-fed with Growth Medium (DMEM, 10% calf serum, and 1% Pen/Strep), using 5 ml per dish. To provide a greater volume of Growth Medium per matrix, HCMs were transferred on day 3 from 60 mm petri dishes to 100 mm petri dishes using flat forceps, and 10 ml of Growth Medium was added per dish. On day 6, medium was aspirated from each HCM, matrices were rinsed with 5 ml of Hank's Balanced Salt Solution (HBSS) and aspirated, and 10 ml of Growth Medium was added to each dish. The time of Growth Medium addition to HCM was noted in order to take a 24 h medium sample the following day (day 7). This rinse and feeding procedure was repeated on days 13 and 20 to provide day 14 and day 21 medium samples. The medium samples were assayed for hGH as indicated below. The HCM were also refed on days 10 and 17 without the rinse step.

Digestion of 3 HCM per ID for cell counts occurred on days 7, 14, and 21 after medium samples were taken. Each matrix was removed from its dish using flat forceps, and excess fluid was carefully blotted from the matrix using an absorbent paper towel. Each matrix was placed in an individual 15 ml conical centrifuge tube containing collagenase IA (1.0 mg/ml in PBS). For matrices seeded with 1-5x10^6 cells, the tubes contained 1 ml of the collagenase solution and for matrices seeded with greater than 5x10^6 cells, the tubes contained 5 ml of the collagenase solution. Each tube was capped tightly, secured in an orbital shaker set at 40 rpm in a tissue culture incubator, and incubated for approximately 1 hour or until digestion was completed. To accelerate digestion, the matrices can be broken up by pipetting at 5 minute intervals. The total
volume of the cell suspension in each tube was measured and 0.1 ml aliquots were removed for counting of the total number of viable and dead cells in each tube using 0.08% trypan blue and an hemacytometer. Production of hGH by HCM at the indicated time points was measured by radioimmunoassay (Nichols Institute) of the 24 h medium samples, as described in Example IX below. Table 2 summarizes the cell numbers for triplicate HCM of each ID, and hGH production by HCM at each ID on days 7 (n = 9 matrices per condition), 14 (n = 6 matrices per condition), and 21 (n = 3 matrices per condition). Values are presented as mean +/- standard deviation. As indicated, the equilibrium density determined at days 14 and 21 for HCM prepared as described above is approximately 7-10 x 10^6 cells per matrix. Per cell hGH production for fully formed matrices at day 21 is similar among the 3 ID levels tested.

<table>
<thead>
<tr>
<th>HCM ID</th>
<th>Cell # Day 7</th>
<th>µg hGH/24h/matrix</th>
<th>µg hGH/24h/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5 x 10⁶</td>
<td>5,331,000 ± 279,347</td>
<td>683 ± 138</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>130 ± 20</td>
</tr>
<tr>
<td>10</td>
<td>10 x 10⁶</td>
<td>9,853,833 ± 858,634</td>
<td>716 ± 137</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>73 ± 6</td>
</tr>
<tr>
<td>15</td>
<td>20 x 10⁶</td>
<td>13,226,000 ± 1,234,410</td>
<td>689 ± 130</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 ± 6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HCM ID</th>
<th>Cell # Day 14</th>
<th>µg hGH/24h/matrix</th>
<th>µg hGH/24h/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>5 x 10⁶</td>
<td>6,534,000 ± 344,525</td>
<td>853 ± 232</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>133 ± 23</td>
</tr>
<tr>
<td>25</td>
<td>10 x 10⁶</td>
<td>9,631,000 ± 875,820</td>
<td>1037 ± 277</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>108 ± 12</td>
</tr>
<tr>
<td>30</td>
<td>20 x 10⁶</td>
<td>10,360,000 ± 706,541</td>
<td>840 ± 280</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>81 ± 16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HCM ID</th>
<th>Cell # Day 21</th>
<th>µg hGH/24h/matrix</th>
<th>µg hGH/24h/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>5 x 10⁶</td>
<td>6,916,167 ± 608,352</td>
<td>730 ± 129</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>105 ± 10</td>
</tr>
<tr>
<td>40</td>
<td>10 x 10⁶</td>
<td>9,884,833 ± 1,475,327</td>
<td>1004 ± 279</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>101 ± 20</td>
</tr>
<tr>
<td>45</td>
<td>20 x 10⁶</td>
<td>10,207,750 ± 2,833,250</td>
<td>872 ± 333</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>83 ± 10</td>
</tr>
</tbody>
</table>
EXAMPLE V

"Standard" collagen matrices (CM) do not include microcarriers. In order to compare CM with HCM, CM were prepared by replacing the volume occupied by microcarriers in HCM with additional soluble collagen, to give a ratio of 1 part 2x DMEM, 1 part calf serum, and 2 parts soluble collagen per CM. A direct comparison of CM with HCM was assessed as follows.

The clone of human fibroblasts stably transfected with the plasmid pXGH302, designated HF165-24, was used. Nine matrices of each type, at each of two ID (1 x 10⁶ and 5 x 10⁶ cells per matrix), were produced. For both CM and HCM, 9 x 10⁶ cells (for 1 x 10⁶ ID) and 45 x 10⁶ cells (for 5 x 10⁶ ID) were resuspended in 9 ml of 2x DMEM + 9 ml of calf serum in 50 ml tubes. For CM, a total of 18 ml of rat tail type I collagen solution (4 mg/ml) was added to each ID set, and matrices were formed as described above in Example I. For HCM, 9 ml collagen microcarriers and 9 ml of rat tail type I collagen solution (4 mg/ml) were added to each ID set, and HCM were formed according to Example I. Matrices were kept in the original 60 mm dish and fed with a volume of 5 ml Growth Medium. Cell numbers per matrix, as well as hGH production per matrix, were determined on days 7, 14, and 30 as described for Example IV. The maximum cell densities (measured on day 14) and hGH production achieved by the 2 types of matrices at the 2 densities are summarized in Table 3. As indicated in the table, the hybrid type of matrix allowed for a higher density of cells and a substantially greater production of hGH per matrix, compared with the standard collagen matrix without microcarriers. These findings also indicate that injectable mixtures not containing microcarriers can also be used to deliver a polypeptide (e.g., a medically useful polypeptide) to a patient. This can be achieved by introducing such mixtures into the patient by any of the routes disclosed herein.
TABLE 3: Comparison of "Standard" Collagen Matrices (CM) and Hybrid Collagen Matrices (HCM) for Maximum Cell Density and hGH Production by Embedded HF165-24 Cells

<table>
<thead>
<tr>
<th>Matrix Type</th>
<th>ID</th>
<th>Maximum cell Density</th>
<th>Maximum hGH Production Per matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>(1 \times 10^6)</td>
<td>(2.1 \times 10^6)</td>
<td>(290 \ \mu g)</td>
</tr>
<tr>
<td>CM</td>
<td>(5 \times 10^6)</td>
<td>(3.3 \times 10^6)</td>
<td>(299 \ \mu g)</td>
</tr>
<tr>
<td>HCM</td>
<td>(1 \times 10^6)</td>
<td>(6.2 \times 10^6)</td>
<td>(983 \ \mu g)</td>
</tr>
<tr>
<td>HCM</td>
<td>(5 \times 10^6)</td>
<td>(10.3 \times 10^6)</td>
<td>(1221 \ \mu g)</td>
</tr>
</tbody>
</table>

EXAMPLE VI

This example describes the production and analysis of precultured hybrid collagen matrices (PCHCM). Cells of the clone of human fibroblasts stably transfected with the plasmid pXGH302 (HF165-24) were seeded onto collagen microcarriers at a ratio of \(2 \times 10^6\) cells per ml of microcarriers, in the following manner. A suspension of \(48 \times 10^6\) cells in 40 ml Growth Medium was obtained from harvested monolayer cultures. 5 ml of this suspension was added to each of four 15 ml graduated tubes containing 6 ml of packed collagen microcarriers, and each cell/microcarrier mixture was transferred to a 125 ml Erlenmeyer flask. An additional 5 ml of cell suspension was added to the cell/microcarrier mixture in the 125 ml Erlenmeyer flask to give a final suspension of \(12 \times 10^6\) cells with 6 ml microcarriers and 10 ml Growth Medium per flask (4 flasks total). The flasks were placed in a tissue culture incubator and swirled gently for approximately 5 seconds each hour for 4 h. After the fourth hour, Growth Medium was added to each flask to the 50 ml mark, and flasks were left undisturbed for 24 h. At 24 h, the microcarriers were transferred from each Erlenmeyer flask into a 250 ml spinner flask, Growth Medium was added to the 150 ml mark of each spinner flask, and flasks were placed on a magnetic stirrer plate set at 50 rpm in a tissue culture incubator. The following day, Growth Medium was added up to the 200 ml mark of each
spinner flask, and flasks were refed 3 times weekly by aspirating medium to the 50 ml mark and adding fresh medium up to 200 ml.

On day 15 of the spinner flask culture, the density of cells per ml microcarriers was determined. A small sample of microcarriers (~0.1 - 0.2 ml) was removed from each flask and placed in pre-weighed 5 ml polystyrene tubes. Excess medium was removed from each tube by aspiration, and the tube containing the microcarrier sample was weighed. One ml of a 2 mg/ml collagenase type IA solution in PBS was added to each tube, and the tubes were covered with parafilm, and placed in a 37°C waterbath. At 15 minute intervals, the tubes containing microcarriers were tapped lightly to disperse clumps. After 1 h, cells were further dissociated by vigorous pipetting with a 5 ml glass pipette. To further dissociate clumps, a solution of 10x trypsin:EDTA was added to give a final trypsin concentration of 1x in the collagenase solution, and the tubes were incubated for an additional 10 minutes. The dissociated cell suspensions were diluted 1:2 with PBS and added to hemacytometer chambers for cell counting. The density of cells per ml microcarriers was calculated using the following formula:

\[
\text{Total # of cells/ml microcarriers} = \\
1000 \text{mg/(mg weight of sample) x (cell# in sample) x 0.5.}
\]

This formula assumes that 1) wet collagen has a specific gravity of 1.0, and therefore the gram weight of collagen in the microcarrier sample equals the collagen volume in milliliters, and 2) half of the wet packed volume of microcarriers is occupied by interstitial volume. The average number of cells per ml microcarriers (n = 4 cultures per condition) for this experiment was 19.2 x 10^6. Microcarriers were removed from each flask and pooled in a 15 ml graduated tube. The entire volume of 6 ml microcarriers containing 19.2 x 10^6 cells per ml microcarriers was used to produce hybrid collagen matrices.

In a 100 ml sterile bottle, 12 ml of modified 2x DMEM, 12 ml of calf serum, 6 ml of empty collagen microcarriers, and the 6 ml of precultured microcarriers were carefully mixed using a 10 ml glass pipette. This mixture was placed on ice, and 12 ml
of ice-cold rat tail Type I collagen was added and mixed carefully using a 10 ml glass pipette. 4 ml of this mixture was added to each of twelve 60 mm petri dishes, and the dishes were placed at 37°C and left undisturbed for 24 h.

The final number of cells per matrix was \(9.6 \times 10^6\), since each matrix was composed of 0.5 ml of microcarriers containing \(19.2 \times 10^6\) cells per ml. These precultured hybrid collagen matrices (PCHCM) were refed after 24 h by aspirating the medium and adding 5 ml of Growth Medium. The PCHCM were refed on days 4, 7, 11, 14, 18, and 20 with 6 ml of Growth Medium. On days 7, 14, and 20, the PCHCM were also rinsed with 4 ml of HBSS prior to addition of medium, and the time of medium addition was noted. On days 8, 15, and 21, a 24 h medium sample was taken for assay of hGH production, and PCHCM were digested to obtain cell counts as follows: A solution of 2 mg/ml collagenase type IA in PBS was added at a volume of 6 ml per 15 ml tube. PCHCM were lifted from dishes with flat forceps and blotted on an absorbent paper towel prior to transfer into a collagenase solution. Tubes containing PCHCM and collagenase solution were secured onto an orbital shaker in a tissue culture incubator, the speed was set to 40 rpm, and PCHCM were allowed to digest for 2 h. After the 2 h incubation, PCHCM were dissociated into single cells by vigorous pipetting with a 5 ml glass pipette. Further dissociation was deemed necessary due to the presence of clumps, and a solution of 10x trypsin:EDTA (0.5% trypsin, 5.3 mM EDTA) was added to give a final trypsin concentration of 1X in the collagenase. The tubes were then incubated for an additional 10 min. Volumes in each tube were noted, and cell suspensions were diluted 2-fold in PBS and placed in a hemacytometer chamber to obtain cell counts. Production of hGH by PCHCM at the indicated time points was measured by radioimmunoassay of the 24 h medium samples, as described in Example VII. Table 4 summarizes the cell numbers for triplicate PCHCM at each time point, and hGH production by PCHCM on days 8 (n = 12 matrices per condition), 15 (n = 9 matrices per condition), and 21 (n = 6 matrices per condition). Values are presented as mean ± standard deviation. As Table 4 shows, these PCHCM support a higher density of cells than the HCM described in Examples IV and V (Tables 2 and 3). The rate of hGH production per matrix and per cell was similar throughout the study period.
TABLE 4
HF165-24 Precultured on Collagen Microcarriers and Embedded in Collagen to Form PCHCM: Cell Number and hGH Production Over Time in Culture.

<table>
<thead>
<tr>
<th>Day</th>
<th>Cell Number</th>
<th>µg hGH/24h/matrix</th>
<th>µg hGH/24h/10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>13,732,000 ± 1,786,565</td>
<td>1264 ± 155</td>
<td>94 ± 10</td>
</tr>
<tr>
<td>10</td>
<td>12,573,000 ± 1,547,133</td>
<td>1317 ± 166</td>
<td>97 ± 14</td>
</tr>
<tr>
<td>21</td>
<td>13,706,000 ± 497,073</td>
<td>1254 ± 135</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EXAMPLE VII

hGH expression was monitored by quantitative hGH measurement with a sandwich radioimmunometric assay (Allegro hGH Assay, Nichols Institute, Cat. No. 40-2205), using conditions recommended by the manufacturer.

In order to determine the rate of hGH production, culture medium was changed 24 hours prior to harvesting the cells for passaging. At the time of passage an aliquot of the culture medium was removed for hGH assay, and the cells were then harvested, counted, and reseeded. hGH levels are calculated after counting the harvested cells, and are expressed as µg hGH/24 hr/10⁶ cells.

EXAMPLE VIII

This example describes in vivo implantation of hybrid collagen matrices (HCM) prepared as described in Example I, as well as standard collagen matrices (CM) prepared as described in Example V.
For subcutaneous implantation of matrices, mice [M. musculus strains N:NIH(S)-nu/nu (nu/nu; Taconic Farms, Germantown, NY) were given an intraperitoneal injection of avertin (solution of 2% w/v 2,2,2-tribromoethanol and 2% v/v 2-methyl, 2-butanol) at a dose of 0.0175 ml/g body weight. Anesthetized mice were placed in lateral recumbency, and the skin prepped with alcohol and Betadine. A 0.5 cm to 1 cm transverse incision was made on the animal's left flank. The subcutaneous space was enlarged by sharp dissection to an area slightly larger than the size of the matrix to be implanted. The matrix was placed horizontally in the subcutaneous space and spread evenly with the use of Millipore forceps. The incision was closed, using stainless steel surgical staples.

Blood was collected by retroorbital bleed after placing the mouse in a large beaker containing methoxyflurane (Pittman-Moore) until light anesthesia was achieved. Serum hGH levels were determined using the commercially available sandwich radioimmunometric assay described above. The assay was performed as described as recommended, except that control serum from untreated mice was used to obtain corrected cpm for generating the standard curve.

HCM and CM were prepared for implantation into nude mice as described in Examples I and V, using hGH-expressing HF165-24 cells. In the first experiment (Experiment 1, Tables 5 and 6), 13 matrices of each type were prepared. HCM were produced with an inoculum density (ID) of 5x10⁶ HF165-24 cells per matrix, and standard collagen matrices (CM) were produced with an ID of 2x10⁶ HF165-24 per matrix. Fewer cells were used to inoculate the CM since these matrices do not support as high a cell density as HCM (see Examples IV and V). In subsequent experiments (Experiments 2 and 3, Tables 5 and 6) only HCM matrices were tested (13 in each of Experiments 2 and 3). Matrices were kept in the original 60 mm dishes and fed with 5 ml of growth medium. After 13 days in culture, all of the dishes were fed with fresh growth medium; 24 h later triplicate matrices of each set were digested for cell counts, and medium samples from all 13 matrices in each set were assayed for hGH.

For Experiment 1, at the time of implantation the average number of cells in the CM was 2.4x10⁶ cells/matrix, while the average number of cells in the HCM was
7.4x10^6 cells/matrix (Table 5). The cell number per matrix was similar to the latter for the HCM prepared in Experiments 2 and 3 (8.9x10^6 and 9.2x10^6 cells per HCM matrix, respectively). Table 5 summarizes the cell number (n = 3 matrices per condition), in vitro hGH production per matrix (n = 13 matrices per condition), and specific production rate (μg/10^6 cells/24 h; n=3 matrices per condition) for each set of in vitro experiments. Values are presented as mean ± standard deviation. As shown in Table 5, HCM supported a higher density of cells and produced a higher level of hGH on the day of implantation than did the CM.

**TABLE 5:** HF165-24 Cell Density and In Vitro hGH Production per Matrix for Collagen Matrices and Hybrid Collagen Matrices on Day of Implantation

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>Cell # on Day of Implantation</th>
<th>μg hGH/24h/matrix</th>
<th>μg hGH/24h/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/CM</td>
<td>2.4 ±0.1 x 10^6</td>
<td>241 ± 33</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>1/HCM</td>
<td>7.4±1.6 x 10^6</td>
<td>983 ± 239</td>
<td>109 ± 16</td>
</tr>
<tr>
<td>2/HCM</td>
<td>8.9±1.5 x 10^6</td>
<td>1399 ± 177</td>
<td>170 ± 27</td>
</tr>
<tr>
<td>3/HCM</td>
<td>9.2±0.9 x 10^6</td>
<td>1279 ± 115</td>
<td>137 ± 19</td>
</tr>
</tbody>
</table>

Eight matrices of each type were implanted into nude mice in Experiment 1, while five HCM were implanted into nude mice in each of Experiments 2 and 3. Serum hGH levels were measured at regular intervals after implantation. The results are shown in Table 6 and Fig. 2. In Experiment 1, HCM-implanted animals maintained substantially higher serum hGH levels than did CM-implanted animals for 186 days post-implantation. Animals implanted with HCM in Experiments 2 and 3 showed similarly high serum hGH levels.
**TABLE 6:** In Vivo Delivery of hGH by Implanted Collagen Hybrid Matrices Containing Transfected Human Skin Fibroblasts

<table>
<thead>
<tr>
<th>Days After Implantation</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CM n=8</td>
<td>HCM n=8</td>
<td>HCM n=5</td>
</tr>
<tr>
<td>7</td>
<td>2.4±0.4</td>
<td>6.8±0.6</td>
<td>13.2±2.0</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>8.5±1.3</td>
<td>8.3±2.0</td>
</tr>
<tr>
<td>15</td>
<td>1.0±0.1</td>
<td>4.2±0.4</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>0.8±0.1</td>
<td>3.7±0.4</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>7.1±2.3</td>
<td>3.6±1.0</td>
</tr>
<tr>
<td>29</td>
<td>0.7±0.1</td>
<td>4.8±1.0</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>0.4±0.1</td>
<td>3.0±0.7</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td>42</td>
<td>0.6±0.1</td>
<td>2.3±0.3</td>
<td>2.9±0.7</td>
</tr>
<tr>
<td>55</td>
<td></td>
<td></td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>56</td>
<td>0.4±0.1</td>
<td>2.7±0.5</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>70</td>
<td>0.3±0.0</td>
<td>1.2±0.2</td>
<td>1.7±0.4</td>
</tr>
<tr>
<td>85</td>
<td>0.4±0.1</td>
<td>1.4±0.2</td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>0.5±0.1</td>
<td>1.2±0.3</td>
<td></td>
</tr>
<tr>
<td>154</td>
<td>0.5±0.1</td>
<td>1.0±0.3</td>
<td></td>
</tr>
<tr>
<td>186</td>
<td>0.6±0.1</td>
<td>1.0±0.5</td>
<td></td>
</tr>
</tbody>
</table>
EXAMPLE IX

General Description of Heparin-Sepharose Hybrid Collagen Matrices (HSHCM)

HSHCM are produced by combining concentrated DMEM, collagen (e.g., rat tail type I or a suitable alternative, for example, human placental type I and III collagen), microcarriers (for example, collagen macroporous microcarriers as described above or porous gelatin microcarriers), heparin-Sepharose beads either uncoated or coated with an angiogenic factor, a cytokine, or a growth factor (for example, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), or platelet derived growth factor (PDGF)), and cells expressing the therapeutic protein. In an alternative embodiment, a combination of cell strains are mixed into the matrix, with one strain expressing the therapeutic protein of interest and the other strain expressing the angiogenic or growth factor. It is also possible to utilize a single strain that expresses both the therapeutic protein and the angiogenic or growth factor. In this embodiment, the heparin-Sepharose bead component may be uncoated or coated with the same or a different growth factor from the one expressed by the incorporated cell strain, or it may be omitted altogether.

Microcarrier Preparation

The preparation of collagen microcarriers for matrix production is as described above in Example I. The preparation of gelatin microcarriers was performed as follows. One gram of dry Cultisphere-GL™ gelatin microcarriers (HyClone Catalog # DG-1001-00) was placed in a 100ml glass bottle containing 50ml of phosphate-buffered saline without calcium or magnesium (PBS). The gelatin microcarriers were allowed to hydrate for at least an hour at room temp. They were sterilized by autoclaving at 121°C for 15 min. The gelatin microcarriers were conditioned for use in matrices by removing the PBS and washing 3x in DMEM + 1% calf serum + 1% Penicillin-Streptomycin (50ml per wash). The gelatin microcarriers were swirled gently and allowed to settle between washes. Conditioned Cultisphere-GL™ gelatin microcarriers can be stored at 4°C prior to use.
**Heparin-Sepharose Bead Preparation**

Heparin coupled to Sepharose® agarose beads provides a solid support for the attachment of heparin-binding growth factors such as bFGF, VEGF, and PDGF. Sepharose® is a highly cross-linked agarose resin manufactured by Pharmacia Biotech and is a registered trademark of the manufacturer. Heparin linked to a different type of support matrix (for example, a polymer) can be used instead of a heparin-agarose bead. Heparin-Sepharose® 6 Fast Flow beads (Pharmacia Biotech Cat #17-0998-03) were washed 3x in dH₂O at 5x the bead volume per wash. The beads were swirled gently after addition of dH₂O and centrifuged at 500xg between each wash step. They were sterilized by autoclaving at 121°C for 20 min. The beads were equilibrated at pH 7.2 - 7.4 by extensive washing in PBS until the pH of the 1:1 bead to PBS slurry was within the range of 7.2-7.4. The beads were stored at 4°C in 1:1 slurry with PBS.

Angiogenic and growth factors were bound to the heparin-Sepharose® beads as follows. Equilibrated beads were placed in an appropriate size sterile tube (for example, a 1.5ml Eppendorf or a 15ml conical tube) and centrifuged at 500xg for 4 min. They were washed 2x in PBS, with a centrifugation step between each wash. The angiogenic or growth factor (e.g., at 50 µg/ml in the case of bFGF) was added to the beads in a volume of PBS equal to the packed bead volume to give a 1:1 bead to PBS slurry, and the binding was performed under rotation (for example, on a Nutator™ platform at room temp. for 1h. The PBS containing any unbound growth factor was removed from the beads by aspiration, and the beads were washed 1x in PBS at a volume equal to the packed bead volume. The PBS was removed, and the beads were placed on ice until matrix formation.

**HSHCM Preparation**

The different components used to prepare HSHCM are listed in Tables 7 and 8. These tables describe production of HSHCM using collagen microcarriers (Table 7) or gelatin microcarriers (Table 8). Each table gives the volume of each component per ml of production medium, as well as the volume necessary for production of 10 x 4ml
HSHCM as an example. (Volumes and concentrations given for matrices hereinbelow refer to the pre-contraction volume and concentration, respectively.) The matrix "pre-mix" consists of 10X DMEM, 7.5% NaHCO₃, 1M HEPES, Penicillin-Streptomycin, L-glutamine, and dH₂O. The pre-mix was generally prepared an hour or two prior to mixing with collagen, cells and the angiogenic factor-coated beads, and kept at 4°C. Cells to be embedded in the HSHCM were harvested and prepared as described in Example I. The cell pellet was resuspended in a volume of growth medium equal to 10% of the total volume of HSHCM production medium as indicated in Tables 7 and 8. The density of cells per ml matrix can vary as appropriate (e.g., 0.1 - 10 x 10⁶ cells per ml matrix). Since the cell suspension was added at a volume that is 10% of the total production medium volume, the number of cells per ml of this suspension was 10x greater than the desired density of cells per ml matrix (e.g. 1 - 100x10⁶ cells per ml). The appropriate volume of microcarriers is placed in a conical tube, and the cell suspension was added to the microcarriers. Using a 10ml plastic pipette, the cells and microcarriers were mixed together and set aside at room temp. for approximately 10 to 15 minutes prior to incorporation into the production medium. The pre-mix was placed on ice and a solution of collagen (e.g., rat tail collagen (RTC)) was added to the chilled pre-mix and mixed thoroughly using a 10ml plastic pipette. The pH of the production medium was then adjusted to approximately 7.8 by addition of 1N NaOH at the volume indicated in Table 7 or 8. The cell/microcarrier mixture was added to the neutralized production medium and mixed using a 10ml plastic pipette. A volume of growth medium equal to the packed volume of heparin-Sepharose beads was added to the beads, and the beads were transferred to the production medium and mixed using a 10ml plastic pipette. A volume of this final HSHCM production medium was added to the appropriate size Petri dish. For example, 4ml of HSHCM production medium is generally added to a 60mm Petri dish, 10ml to a 100mm Petri dish, and 30ml to a 150mm Petri dish. Varying the ratio of production medium volume to dish surface area can modify the thickness of the HSHCM. The dishes containing the HSHCM production medium were transferred to an incubator with a temperature of 37°C, humidity of 95 to 98%, and a CO₂ level of 5%. One hour later, the dishes were removed
from the incubator and examined. If the HSHCM production medium had gelled, the HSHCM were fed by addition of 5ml of growth medium. Otherwise, the dishes were returned to the incubator for an additional hour or until polymerization was complete, at which time the HSHCM were fed by addition of 5 ml of growth medium. The growth medium was supplemented with 10 - 100 ng/ml of the same growth factor or angiogenic factor used to coat the heparin-Sepharose beads of the HSHCM.

Table 7. HSHCM Production Media Formulation: Collagen Microcarrier Configuration.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (per ml)</th>
<th>Total Vol. for 10 x 4ml matrices</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X DMEM</td>
<td>0.1ml</td>
<td>4.0ml</td>
<td>1X</td>
</tr>
<tr>
<td>7.5% NaHCO₃</td>
<td>0.012ml</td>
<td>0.48ml</td>
<td>0.9g/L</td>
</tr>
<tr>
<td>1M HEPES</td>
<td>0.0025ml</td>
<td>0.1ml</td>
<td>2.5mM</td>
</tr>
<tr>
<td>Pen/Strep (100X stock)</td>
<td>0.01ml</td>
<td>0.4ml</td>
<td>1%</td>
</tr>
<tr>
<td>L-glutamine (200mM stock)</td>
<td>0.01ml</td>
<td>0.4ml</td>
<td>2mM</td>
</tr>
<tr>
<td>dH₂O</td>
<td>0.16ml</td>
<td>6.4ml</td>
<td>16%</td>
</tr>
<tr>
<td>RTC (4mg/ml, 0.02N acetic acid)</td>
<td>0.25ml</td>
<td>10ml</td>
<td>1mg/mL</td>
</tr>
<tr>
<td>1N NaOH (to pH 7.4-7.8)</td>
<td>0.005ml</td>
<td>0.2ml</td>
<td>0.5%</td>
</tr>
<tr>
<td>Collagen microcarriers*</td>
<td>0.25ml</td>
<td>10ml</td>
<td>25%</td>
</tr>
<tr>
<td>Cell suspension*</td>
<td>0.1ml</td>
<td>4.0ml</td>
<td>10%</td>
</tr>
<tr>
<td>Heparin-Sepharose beads (as a 1:1 slurry in DMEM + 10% calf serum)*</td>
<td>0.1ml</td>
<td>4.0ml</td>
<td>5.0% dry weight</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1.0ml</td>
<td>40.0ml</td>
<td></td>
</tr>
</tbody>
</table>

*Added to the production medium after pH is adjusted
Table 8. HSHCM Production Media Formulation: Gelatin Microcarrier Configuration.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (per ml)</th>
<th>Total Vol. for 10 x 4ml matrices</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X DMEM</td>
<td>0.1ml</td>
<td>4.0ml</td>
<td>1X</td>
</tr>
<tr>
<td>7.5% NaHCO₃</td>
<td>0.012ml</td>
<td>0.48ml</td>
<td>0.9g/L</td>
</tr>
<tr>
<td>1M HEPES</td>
<td>0.0025ml</td>
<td>0.1ml</td>
<td>2.5mM</td>
</tr>
<tr>
<td>Pen/Strep (100X stock)</td>
<td>0.01ml</td>
<td>0.4ml</td>
<td>1%</td>
</tr>
<tr>
<td>L-glutamine (200mM stock)</td>
<td>0.01ml</td>
<td>0.4ml</td>
<td>2mM</td>
</tr>
<tr>
<td>dH₂O</td>
<td>0.15ml</td>
<td>6.0ml</td>
<td>15%</td>
</tr>
<tr>
<td>RTC (4mg/ml, 0.02N acetic acid)</td>
<td>0.438ml</td>
<td>17.5ml</td>
<td>1.75mg/ml</td>
</tr>
<tr>
<td>1N NaOH (to pH 7.4-7.8)</td>
<td>0.015ml</td>
<td>0.6ml</td>
<td>1.5%</td>
</tr>
<tr>
<td>Gelatin microcarriers*</td>
<td>0.0625ml</td>
<td>2.5ml</td>
<td>6.25%</td>
</tr>
<tr>
<td>Cell suspension*</td>
<td>0.1ml</td>
<td>4.0ml</td>
<td>10%</td>
</tr>
<tr>
<td>Heparin-Sepharose beads (as a 1:1 slurry in DMEM + 10% calf serum)*</td>
<td>0.1ml</td>
<td>4.0ml</td>
<td>5.0% dry weight</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1.0ml</td>
<td>40.0ml</td>
<td></td>
</tr>
</tbody>
</table>

* Added to the production medium after pH is adjusted

In Vitro Maintenance and Evaluation of HSHCM

HSHCM were maintained in culture as described above for HCM in Example IV. The volume of growth medium used to feed the matrices is usually the maximum volume that can be added to each dish size, for example, 10ml per 60mm dish, 20-30ml per 100mm dish, and 100 - 150ml per 150mm dish. Matrix size and cell number per matrix were determined as described for the HCM.
EXAMPLE X

This example describes in vivo implantation of HSHCM prepared as described in Example IX. HSHCM containing either uncoated heparin-Sepharose beads or heparin-Sepharose beads coated with bFGF (50μg/ml packed beads; 10μg total bFGF/matrix) were prepared as described in Table 7. Each 4ml matrix was formed with 5 x 10^6 cells of HP743 B1-35, a human foreskin fibroblast clone containing plasmid pXF8.198 (Fig. 3) and expressing hFVIII at a level between 20,000 - 30,000 mU/24h/ 10^6 cells. The HSHCM were maintained in culture for 2 days prior to implantation. For subcutaneous implantation of matrices, Rag-2 mice (129S6/SvEvTac-[KO]Rag2, Taconic Farms) were anesthetized and prepped as described in Example VIII. The implant site was shaved and disinfected with alcohol and Betadine. A 2 cm incision was made in the cutaneous layer on the left side of the animal, inferior and caudal to the ribs, and the fascia between the cutaneous and external oblique muscle layer was cleared and enlarged using sterile blunt scissors. HSHCM were prepared for implantation by washing 2x in 10 ml of PBS. Each matrix was folded into quarters using sterile spoon-shaped spatulas, and inserted into the cleared cutaneous space using a sterile spatula. The incision was closed using wound clips.

Blood was collected as described in Example VIII. Plasma hFVIII was determined using a hFVIII ELISA based on two mouse monoclonal antibodies [Hornsey et al. (1992) Transfus. Med. 2(3):223-229]. The estimated number of cells per matrix was determined by digesting and counting recovered cells from HSHCM set aside for this purpose. HSHCM containing uncoated heparin-Sepharose beads had an average of 2.8 x 10^6 cells per matrix and HSHCM containing bFGF-coated heparin-Sepharose beads had an average of 3.1 x 10^6 cells per matrix (n = 3 matrices per condition). The in vitro production of hFVIII from each matrix type on the day of implantation was 71,165 mU/24h and 85,657 mU/24h from HSHCM containing uncoated and bFGF-coated beads, respectively (n = 3 matrices per condition). As shown in Fig. 4, HSHCM containing bFGF-coated heparin-Sepharose beads led to a significantly higher plasma level of hFVIII detected in the host mice as compared to HSHCM without the growth
factor (n = 10 mice per condition). There was no detectable hFVIII on day 0, and the peak plasma level occurred on day 14.

The following examples describe additional studies involving subcutaneous implantation of HSHCM (prepared as described in Example IX) into Rag-2 mice, and utilize the HF743-B1-35 hFVIII secreting cell clone introduced above. Time of implantation and implant procedures are identical to that described in Example VIII unless otherwise indicated.

**EXAMPLE XI**

HSHCM containing either uncoated heparin-Sepharose beads or heparin-Sepharose beads coated with bFGF (50µg/ml packed beads, 10µg total bFGF/matrix), and either collagen microcarriers or gelatin microcarriers, were prepared as described in Tables 7 and 8, respectively. The average number of cells per matrix and hFVIII production per matrix on the day of implantation (n = 3 matrices per condition) are listed in Table 9.

**Table 9.** Summary of cell number and hFVIII production per HSHCM on the day of implantation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell # (x 10⁶)</th>
<th>hFVIII (mU/24h/HSHCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen microcarriers &amp; uncoated heparin-Sepharose beads</td>
<td>4.2</td>
<td>84,989</td>
</tr>
<tr>
<td>Gelatin microcarriers &amp; uncoated heparin-Sepharose beads</td>
<td>3.9</td>
<td>94,407</td>
</tr>
<tr>
<td>Collagen microcarriers &amp; bFGF-coated heparin-Sepharose beads</td>
<td>4.3</td>
<td>130,612</td>
</tr>
<tr>
<td>Gelatin microcarriers &amp; bFGF-coated heparin-Sepharose beads</td>
<td>3.5</td>
<td>107,683</td>
</tr>
</tbody>
</table>
Subcutaneous (SC) cell controls were included in this experiment and were prepared as follows. Cells were harvested by trypsinization in the same manner as for preparation of HCM. The trypsinized cells were counted, centrifuged at 500xg, resuspended in Hank's Buffered Saline Solution (without calcium and magnesium), centrifuged, and resuspended in a volume of PBS to give 100 x 10^6 cells/ml based on the original cell count after harvest. The cells in the cell/PBS slurry were counted, and the density of cells in the suspension was adjusted to 50 x 10^6 cells/ml PBS. For each injection, a total of 0.15ml of cell slurry was aspirated into a 1cc Glasspak syringe and the syringe was capped with a 22G/1 inch needle. The cell slurry was injected SC into the left side of the mouse, inferior and caudal to the ribs. Taking into consideration the void volume of the needle, a total of 0.1ml, or 5 x 10^6 cells, was injected into each control animal (n = 5 mice per condition).

Fig. 5 shows that the HSHCM containing the gelatin microcarriers and bFGF-coated heparin-Sepharose beads led to a significantly higher level of hFVIII compared to the other conditions (n = 10 mice per condition). The expression pattern was similar to that observed in Example X, with no detectable hFVIII until day 7, and highest expression at day 14. There was no detectable hFVIII in the plasma of the mice receiving SC cell injections at any time point after day 1 post-implantation.

**EXAMPLE XII**

HSHCM containing heparin-Sepharose beads either uncoated or coated with bFGF at 12.5, 25, or 50μg/ml packed beads were prepared using the collagen microcarrier formulation (Table 7). The total amount of bFGF per matrix after incorporation of the coated beads was estimated to be approximately 2, 4, and 10μg. The average number of cells per matrix and hFVIII production per matrix on the day of implantation (n = 3 matrices per condition) are summarized in Table 10.
Table 10. Summary of cell number and hFVIII production per HSHCM on the day of implantation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell # (x 10^6)</th>
<th>hFVIII (mU/24h/HSHCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated heparin-Sepharose beads</td>
<td>3.8</td>
<td>62,956</td>
</tr>
<tr>
<td>BFGF at 2μg/HSHCM</td>
<td>7.0</td>
<td>92,836</td>
</tr>
<tr>
<td>BFGF at 4μg/HSHCM</td>
<td>10.9</td>
<td>80,976</td>
</tr>
<tr>
<td>BFGF at 10μg/HSHCM</td>
<td>8.2</td>
<td>97,964</td>
</tr>
</tbody>
</table>

Fig. 6 demonstrates that plasma levels of hFVIII are significantly higher from matrices containing 10μg of bFGF as compared to those containing 4μg and 2μg of bFGF (n = 5 mice per condition). The pattern of expression was similar to that observed in Examples X and XI.

**EXAMPLE XIII**

HSHCM containing heparin-Sepharose beads either uncoated, coated with bFGF, coated with VEGF, or coated with both bFGF and VEGF were prepared according to the gelatin microcarrier formulation (Table 8). For the set of HSHCM containing beads coated with both growth factors, each matrix received 0.1ml of beads coated with bFGF and 0.1ml of beads coated with VEGF in order to keep the bead volume consistent with previous experiments. The coating concentration of each growth factor for this condition was 100μg/ml packed beads, resulting in a total of approximately 10μg of each growth factor per HSHCM. The average number of cells per matrix and hFVIII production per matrix on the day of implantation (n = 3 matrices per condition) are summarized in Table 11.
Table 11. Summary of cell number and hFVIII production per HSHCM on the day of implantation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell # (x 10^6)</th>
<th>hFVIII (mU/24h/HSHCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated heparin-Sepharose beads</td>
<td>3.7</td>
<td>101,048</td>
</tr>
<tr>
<td>HSHCM + bFGF</td>
<td>3.5</td>
<td>128,654</td>
</tr>
<tr>
<td>HSHCM + VEGF</td>
<td>3.4</td>
<td>76,152</td>
</tr>
<tr>
<td>HSHCM + bFGF + VEGF</td>
<td>4.9</td>
<td>127,450</td>
</tr>
</tbody>
</table>

Intraomental (IO) implantation controls were included in order to compare the subcutaneous delivery of hFVIII from HSHCM with another implantation method that has proven successful for the delivery of hFVIII in the mouse model. IO controls were prepared as follows. Cells were harvested by trypsinization in the same manner as for preparation of HCM. The trypsinized cells were counted, centrifuged at 500xg, resuspended in Hank's Buffered Saline Solution (without calcium and magnesium), recentrifuged, and resuspended in a volume of PBS to give 15 x 10^6 cells/ml based on the original cell count after harvest. The cells in the cell/PBS slurry were counted, and a volume of cell suspension equal to 5 x 10^6 cells was distributed into sterile 1.5ml Eppendorf tubes and centrifuged at 500xg for 4 min in a microcentrifuge. The tubes containing the cell pellet and PBS were placed on ice, and each cell pellet was implanted into the omental recess of the Rag-2 mouse in the following manner. Prior to implantation of cells, mice were anesthetized with 1.25% Avertin and placed in lateral recumbency. The left flank between the ribs and stifle was wiped with an alcohol pad and prepped with Betadine. A small (0.5 cm to 1.0 cm) incision was made posterior to the ribs and ventral to the spine. The spleen was exposed and gently exteriorized. Cells were injected along the axis of the spleen upon the cranial and medial aspect and within the thin membrane adjacent to the hilar surface of the spleen. The spleen was replaced in the abdominal cavity, and the incision closed.

Fig. 7 demonstrates that the plasma hFVIII levels are significantly higher from HSHCM containing a combination of bFGF and VEGF as compared to the other
conditions at days 14 and 21 (n = 5 mice per condition). The injection of cells IO led to a significantly higher plasma hFVIII level on day 1, but by the next time point (day 7), the level observed in IO controls fell below that obtained with the HSHCM implants containing growth factors (n = 5 mice per condition).

EXAMPLE XIV

HSHCM containing a combination of 2 fibroblast cell clones, HF743 B1-35 expressing hFVIII and HF811-M15 containing plasmid pXVEGF.1 (Fig. 8) and expressing VEGF [Kock et al., Science 246:1309-1312, 1989], were prepared for implantation. The HF811-M15 clone was producing an average of 85 ng VEGF/24h/10^6 cells at the time of implantation. The matrices were prepared according to the collagen microcarrier formulation (Table 7). The heparin-Sepharose bead component was uncoated. Two sets of HSHCM were prepared with one or the other clone at 5 x 10^6 cells per matrix. An additional two sets were prepared with a constant number of HF743 B1-35 cells (5 x 10^6) and an additional 1 x 10^6 or 2.5 x 10^6 of HF811-M15 cells. Table 12 summarizes the different conditions and production levels of hFVIII and VEGF for each condition (n = 5 matrices per condition).
Table 12. Summary of hFVIII and VEGF production per HSHCM on day of implantation.

<table>
<thead>
<tr>
<th>Condition (cells/matrix)</th>
<th>hFVIII (mU/24h)</th>
<th>VEGF (ng/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF743 B1-35 (5 x 10^6)</td>
<td>65,442</td>
<td>n/a</td>
</tr>
<tr>
<td>HF743 B1-35 (5 x 10^6) + HF811-M15 (1 x 10^6)</td>
<td>79,828</td>
<td>47</td>
</tr>
<tr>
<td>HF743 B1-35 (5 x 10^6) + HF811-M15 (2.5 x 10^6)</td>
<td>63,876</td>
<td>86</td>
</tr>
<tr>
<td>HF811-M15 (5 x 10^6)</td>
<td>n/a</td>
<td>82</td>
</tr>
</tbody>
</table>

Plasma hFVIII levels over time for each HSHCM condition are illustrated in Fig. 9. The set of HSHCM containing 2.5 x 10^6 HF811-M15 cells led to the highest hFVIII plasma levels, which peaked on day 14 and declined until day 42, at which time no hFVIII was detectable. The HSHCM containing 1 x 10^6 HF811-M15 cells led to plasma levels of hFVIII slightly lower than the set containing 2.5 x 10^6 HF811-M15, but significantly higher than the HSHCM without the VEGF-producing cells, indicating that VEGF produced a dose-dependent effect on the delivery of hFVIII by the implant.

The amount of VEGF in the plasma samples was determined by ELISA (R&D Systems VEGF Immunoassay Kit). The results are summarized in Table 13. There was a decline in detectable VEGF from day 14 to day 28 in plasma from mice implanted with HSHCM made with either 2.5 x 10^6 or 5 x 10^6 HF811-M15 cells. VEGF levels were not detectable in the plasma of animals implanted with HSHCM made without or with 1 x 10^6 HF811-M15 cells. Plasma samples for day 1 and 7 were not available for assay, but the data suggest that the VEGF level peaked at some point between time of implantation and day 14.
Table 13. Summary of VEGF expression in the plasma of Rag-2 mice implanted with HSHCM containing different numbers of HF811-M15 cells.

<table>
<thead>
<tr>
<th>Condition (cells/matrix)</th>
<th>Plasma VEGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>HF743 B1-35 (5 x 10^6)</td>
<td>0</td>
</tr>
<tr>
<td>HF743 B1-35 (5 x 10^6) + HFB11-M15 (1 x 10^6)</td>
<td>0</td>
</tr>
<tr>
<td>HF743 B1-35 (5 x 10^6) + HFB11-M15 (2.5 x 10^6)</td>
<td>6.5</td>
</tr>
<tr>
<td>HF811-M15 (5 x 10^6)</td>
<td>89</td>
</tr>
</tbody>
</table>

EXAMPLE XV

General Description of Injectable Heparin-Sepharose Hybrid Collagen Matrix Mixtures (I-HSHCMM)

I-HSHCMM are prepared by mixing together concentrated DMEM (without phenol red), rat tail type I collagen (or a suitable alternative, for example, human placental type I and/or III collagen); porous microcarriers (for example, collagen macroporous microcarriers or porous gelatin Cultispheres); optional heparin-Sepharose beads either uncoated or coated with an angiogenic or growth factor (for example, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), or platelet derived growth factor (PDGF)); and cells expressing the therapeutic protein. In an alternative embodiment, a combination of cell strains are mixed into the collagen solution, with one strain expressing the therapeutic protein of interest and the other strain expressing the angiogenic or growth factor. It is also possible to utilize a single strain that expresses both the therapeutic protein and the angiogenic or growth factor. In this
embodiment, the heparin-Sepharose bead component may be uncoated or coated with the same or a different growth factor from the one expressed by the incorporated cell strain, or it may be omitted altogether. Injectable matrix mixtures of the invention include those without collagen or any of the alternatives disclosed herein. Furthermore, mixtures without a source of angiogenic factors or growth factors are included in the invention. It is also understood that the mixtures may lack microcarriers, but generally such mixtures will include an agent such as an angiogenic factor, a growth factor, or ascorbic acid. While not a requirement, such agents can be associated with any of the solid substrates described herein and these solid substrates may function as microcarriers to which the cells adhere, and also as reservoirs of relevant agents.

Microcarrier Preparation and Heparin-Sepharose Bead Preparation

Microcarriers and Heparin-Sepharose beads were prepared as described above in Examples I and IX. Serum proteins were removed by 3-5 washes with serum-free PBS prior to formation of the mixtures.

I-HSHCMM Preparation

The different components used to prepare I-HSHCMM are listed in Table 14, which describes preparation of I-HSHCMM using Cultispheres as the microcarrier. The table gives the volume of each component per mL of production medium, as well as the volume necessary for production of 10 x 1mL and 10 x 2mL I-HSHCMM as an example. The matrix "pre-mix" consists of 4X DMEM without phenol red, 7.5% NaHCO₃, 1M HEPES, Penicillin-Streptomycin, L-glutamine, and dH₂O. The pre-mix is usually prepared an hour or two in advance and kept at 4°C. Cells to be injected along with the matrix components are harvested from tissue culture vessels and centrifuged at 500xg for 10 min to obtain a cell pellet. The cell pellet is washed 1x in PBS, the PBS is removed, and the pellet is resuspended in 1X DMEM without phenol red in a volume equal to 10% of the total volume of the I-HSHCMM production medium as indicated in Table 14. The density of cells per mL matrix production medium can vary as
appropriate (e.g. 0.1 - 10 × 10⁶ cells per mL injectable matrix). Since the cell suspension is added at a volume that is 10% of the total production medium volume, the number of cells per mL of this suspension should be 10x greater than the desired density of cells per mL matrix mixture (e.g., 1 - 100 × 10⁶ cells per mL). The appropriate volume of microcarriers (e.g., Cultispheres) is placed in a conical centrifuge tube, and the cell suspension is added to the microcarriers. Using a 10mL glass pipette, the cells and microcarriers are mixed together and set aside at room temp. for approximately 10 to 15 minutes prior to incorporation into production medium. The pre-mix is placed on ice and a solution of collagen (e.g. rat tail collagen (RTC)) is added to the chilled pre-mix and mixed thoroughly using a 10mL glass pipette. The production medium is then adjusted to a pH of approximately 7.8 by addition of 1N NaOH at the volume indicated in Table 14. The cell/microcarrier mixture is pelleted by centrifugation at 500g for 5 min, the supernatant is aspirated, and a volume of DMEM without phenol red is added to the tube to achieve a final volume equal to the volume of the cell suspension plus the volume of microcarriers as listed in Table 14. The cell/microcarrier suspension is added to the neutralized production medium and mixed using a 10mL glass pipette. A volume of DMEM without phenol red equal to the packed volume of heparin-Sepharose beads is added to the beads, and the beads are transferred to the neutralized production medium and mixed using a 10mL glass pipette. The complete production medium is kept on ice until immediately prior to injection, at which time it is loaded into an appropriate size syringe (e.g., 10cc). After loading, the syringe is capped with a needle (e.g., 16 gauge) and injected into the desired site (e.g., subcutaneous).
Table 14. Injectable HSHCMM Production Media Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (per mL)</th>
<th>Total Vol. for 10 x 1mL solutions</th>
<th>Total Vol. for 2mL solutions</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X DMEM</td>
<td>0.25mL</td>
<td>2.5mL</td>
<td>5.0mL</td>
<td>1X</td>
</tr>
<tr>
<td>7.5% NaHCO₃</td>
<td>0.012mL</td>
<td>0.12mL</td>
<td>0.24mL</td>
<td>0.9g/L</td>
</tr>
<tr>
<td>1M HEPES</td>
<td>0.0025mL</td>
<td>0.025mL</td>
<td>0.05mL</td>
<td>2.5mM</td>
</tr>
<tr>
<td>dH₂O</td>
<td>0.0315mL</td>
<td>0.315mL</td>
<td>0.63mL</td>
<td>3.15%</td>
</tr>
<tr>
<td>RTC (4mg/mL, 0.02N acetic acid)</td>
<td>0.438mL</td>
<td>4.38mL</td>
<td>8.76mL</td>
<td>1.75mg/mL</td>
</tr>
<tr>
<td>1N NaOH (to pH 7.4-7.8)</td>
<td>0.004mL</td>
<td>0.04mL</td>
<td>0.08mL</td>
<td>0.4%</td>
</tr>
<tr>
<td>Cultispheres*</td>
<td>0.0625mL</td>
<td>0.625mL</td>
<td>1.25mL</td>
<td>6.25%</td>
</tr>
<tr>
<td>Cell suspension*</td>
<td>0.1mL</td>
<td>1.0mL</td>
<td>2.0mL</td>
<td>10%</td>
</tr>
<tr>
<td>Heparin-Sepharose beads (as a 1:1 slurry in DMEM without phenol red)*</td>
<td>0.1mL</td>
<td>1.0mL</td>
<td>2.0mL</td>
<td>5.0% dry weight</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1.0mL</td>
<td>10.0mL</td>
<td>20.0mL</td>
<td></td>
</tr>
</tbody>
</table>

* Added to the production medium after pH is adjusted

In Vitro Assessment of Gelled Mixture

A volume of the final I-HSHCMM production medium is added to the appropriate size Petri dish via injection from a syringe through a 16G needle as described above. As an example, 2mL of I-HSHCMM production medium are added to a 35mm Petri dish, 4mL to a 60mm Petri dish, 10mL to a 100mm Petri dish, and 30mL to a 150mm Petri dish. Varying the ratio of production medium volume to dish surface area can modify the thickness of the gelled HSHCM. The dishes containing the I-HSHCMM production medium are then transferred to an incubator with a temperature of 37°C, humidity of 95 to 98%, and CO₂ level of 5%. One hour later, the dishes are removed from the incubator and examined. If the I-HSHCMM production medium has gelled, the HSHCM are fed by addition of 5mL of DMEM without phenol red,
otherwise, the dishes are returned to the incubator for an additional hour or until polymerization is complete.

HSHCM are maintained in culture for the desired amount of time. The volume of culture medium used to feed the matrices is usually the maximum volume that can be added to each dish size, for example, 5mL per 35mm dish, 10mL per 60mm dish, 20-30mL per 100mm dish, and 100-150mL per 150mm dish. The cell number, cell viability per matrix, and level of polypeptide production are determined as described in detail in Example IV.

**EXAMPLE XVI**

This example describes the *in vivo* injection of the I-HSHCMM prepared as described in Example XV. I-HSHCMM containing heparin-Sepharose beads coated with bFGF at 100μg/mL packed beads for a 2mL injection volume and 200μg/mL packed beads for a 1mL injection volume (10μg total bFGF delivered per implant) was prepared as described in Table 14. For each injection volume, HF743 B1-35, a human foreskin fibroblast clone containing plasmid pXF8.198 (Fig. 3) and expressing hFVIII at levels between 20,000 - 30,000 mU/24h/10^6 cells, was incorporated, with microcarriers, to achieve a total of 5 x 10^6 cells per injection. For subcutaneous introduction of matrices, Rag-2 mice (129S6/SvEvTac-[KO]Rag-2, Taconic Farms) were anesthetized and prepped as described in Example VIII. The implant site, located on the left side of the animal inferior and caudal to the ribs, was shaved and disinfected with alcohol and Betadine. Immediately prior to injection, the complete I-HSHCMM was loaded into a 10cc syringe at a volume slightly greater than the desired injection volume to compensate for the void volume of the needle. The loaded syringe was capped with a 16G needle and the I-HSHCMM was immediately injected into the subcutaneous site. The injected solution spread out within the space between the cutaneous and external oblique muscle. Slight pressure was applied to the point of injection and the needle was slowly pulled away from the skin. The injected solution began to solidify within a few
seconds after injection. The mice were placed on a heat pad immediately after injection to prevent possible hypothermia.

Blood was collected as described in Example VIII and plasma hFVIII was determined using a hFVIII ELISA based on two mouse monoclonal antibodies as described in Example X. The estimated number of cells per gelled matrix was determined by digesting and counting recovered cells from HSHCM formed in Petri dishes (2mL in 35mm dish) and set aside for this purpose. The HSHCM gels were kept in culture for 2 days prior to assessment of cell number and viability, and were maintained in serum-free DMEM as described in Example XV. Gelled HSHCM (n = 3 matrices per condition) had an average of $3.51 \times 10^6 \pm 0.8 \times 10^6$ cells per matrix. The viability of the recovered cells was 84.5 ± 2.4%. The *in vitro* production of hFVIII from the gelled matrices was $68,851 \pm 199$ mU/24h. Fig. 10 depicts the plasma levels of hFVIII over time after subcutaneous injection of the HSHCM production medium into *Rag-2* mice. Error bars indicate the standard error of the mean (n = 5 mice per injection volume). Peak expression occurred one day after injection.

**EXAMPLE XVII**

This example describes the intraomental injection into *Nude* mice of rabbit fibroblasts expressing hFVIII, either alone (i.e., without microcarriers) or in combination with heparin-Sepharose beads coated with growth factors. Heparin-Sepharose beads were coated with either bFGF (1μg/mL packed beads) or VEGF (2μg/mL packed beads) as described in Example XV. Coated beads were distributed into 1.5mL polypropylene Eppendorf tubes to achieve a volume of 50μL packed beads per tube. Excess PBS was removed from the beads by wicking with sterile absorbent gauze. Cells of RF261 B2-106, a rabbit skin fibroblast clone containing the plasmid pXF8.186 (Fig. 11) and expressing hFVIII at levels between 20,000 - 25,000 mU/24h/10^6 cells, were harvested from tissue culture flasks by conventional methods. Trypsinized fibroblasts were centrifuged to a pellet (500xg for 10 min), washed with Hank’s Balanced Salt Solution (HBSS), centrifuged, and resuspended in a volume of PBS to achieve a cell density of
approximately $15 \times 10^6$ cells/mL. A volume equaling $5 \times 10^6$ cells was added either to empty 1.5 mL Eppendorf tubes or to the tubes containing 50μL of heparin-Sepharose beads, and the suspensions were centrifuged at 500xg for 3 min. The final volume in the tubes containing the beads and cells was adjusted to 100μL by removing PBS. Twelve replicates were prepared for each condition. The tubes containing the cell pellets and cell/bead slurries were placed on ice and transported to the Animal Facility.

For intraomental (IO) injection of cells and beads, *Nude* mice (NIH Swiss *Nude* (nu/nu, Tac:N:NIH(S)-Hfh11nu, Taconic Farms) were anesthetized and prepped as described in Example VIII. Animals were placed in lateral recumbency and the left flank between the ribs and stifle wiped with an alcohol pad and prepped with Betadine. A small (0.5 cm to 1.0 cm) incision was made posterior to the ribs and ventral to the spine. The spleen was exposed and gently exteriorized. Using a 20-gauge cannula, cell pellets and cell/bead slurries were injected along the axis of the spleen upon the cranial and medial aspect and within the thin membrane adjacent to the hilar surface of the spleen. The spleen was replaced in the abdominal cavity, and the incision closed.

Methods for blood collection and determination of hFVIII were as described in Example VIII. Fig. 12 shows the plasma levels of hFVIII over time after the injection of cells alone, cells and heparin-Sepharose beads coated with bFGF (50ng total per implant), and cells and heparin-Sepharose beads coated with VEGF (100ng total per implant). Error bars indicate the standard error of the mean (n = 12 mice per condition). Peak expression occurred on day 1. The cell/bead/VEGF combination led to significantly higher hFVIII levels between days 28 and 70 compared to the other conditions.

**EXAMPLE XVIII**

This example describes the intraomental injection into *Nude* mice of rabbit fibroblasts expressing hFVIII, either alone or in combination with heparin-Sepharose beads coated with growth factors.
The heparin-Sepharose beads were either uncoated or coated with bFGF (10µg/mL packed beads) or VEGF (2µg/mL packed beads) as described in Example IX. The uncoated and growth factor-coated beads were distributed into tubes as described in Example XVII. Cells of RF302 B2-383, a rabbit skin fibroblast clone containing the plasmid pXF8.186 and expressing hFVIII at levels between 20,000 - 25,000 mU/24h/10^6 cells, were harvested from tissue culture flasks by conventional methods. The cells were processed and distributed into empty Eppendorf tubes or tubes containing heparin-Sepharose beads as described in Example XVII. Intraomental injection, blood collection, and hFVIII measurement were carried out as described in Example XVII.

Fig. 13 depicts the plasma levels of hFVIII over time after the injection of cells alone, cells and uncoated heparin-Sepharose beads, cells and heparin-Sepharose beads coated with bFGF (500ng total per implant), and cells and heparin-Sepharose beads coated with VEGF (100ng total per implant). Error bars indicate the standard error of the mean (n = 12 mice per condition). Peak hFVIII expression occurred on day 1, and was significantly greater for the bFGF set compared to all other conditions on days 1, 7, and 14. On day 7, the cell/bead/VEGF combination led to significantly higher hFVIII levels compared to the cells alone and the cell/uncoated bead conditions.

**EXAMPLE XIX**

This example describes the intraomental injection into Nude mice of rabbit fibroblasts expressing hFVIII, either with macroporous collagen microcarriers or with microcarriers in combination with heparin-Sepharose beads coated with growth factors.
Heparin-Sepharose beads were either uncoated or coated with bFGF (10μg/mL packed beads) or VEGF (2μg/mL packed beads) as described in Example IX. The microcarriers and cells were prepared as follows. Macroporous collagen microcarriers (Cellex Biosciences) were conditioned as described in Example I. A slurry of microcarriers (1:1 volume of microcarriers:conditioning medium) was prepared, and 0.1mL aliquots were added to 1.5mL Eppendorf tubes. These tubes were centrifuged at 500xg for 4 min, aspirated, and washed 2x with PBS. The PBS was removed and the tubes were stored on ice. A 0.1mL slurry of heparin-Sepharose beads (uncoated, coated with bFGF, or coated with VEGF) in PBS at a 1:1 ratio (vol/vol) was added to the tubes containing microcarriers and the bead/microcarrier mixtures were centrifuged at 500xg for 4 min. The PBS was removed from each tube and the bead/microcarrier pellets were stored on ice. RF302 B2-383 cells were processed as described in Example XVII. A volume of cell suspension containing 5 x 10^6 cells was added to each tube containing either microcarriers alone or microcarriers and heparin-Sepharose beads, and the tubes were centrifuged at 500xg for 4 min. The supernatant was removed from each tube and 10μl of PBS was added. Tubes were placed on ice and transported to the Animal Facility. Intraomental injection, blood collection, and hFVIII measurement were carried out as described in Example XVII.

Fig. 14 shows the plasma levels of hFVIII over time after injection of cells and microcarriers, cells and microcarriers and uncoated heparin-Sepharose beads, cells and microcarriers and beads coated with bFGF (500ng total per implant), and cells and microcarriers and beads coated with VEGF (100ng total per implant). Error bars indicate the standard error of the mean (n = 12 mice per condition). Peak hFVIII expression occurred on day 1, and was significantly greater for the bFGF set compared to the other conditions on days 1, 7, and 14.

**EXAMPLE XX**

Keratinocytes are epidermal cells of the skin, and are normally contiguous with fibroblasts in this tissue. These two cell types depend on one another for the proper
nutrients, differentiation signals, and production and maintenance of surrounding extracellular matrix. The addition of keratinocytes to any of the injectable hybrid collagen matrix mixtures described herein which contain fibroblasts can benefit the fibroblasts within the matrices formed from the mixtures in a manner similar to what occurs in the skin. The keratinocytes and fibroblasts can be isolated from the same skin biopsy. The keratinocytes can be added simultaneously with the fibroblasts at the time of mixture formation, prior to introduction into an animal. The keratinocytes within the matrix can be stimulated to differentiate, or not, depending upon the growth medium formulation and culture conditions. For example, an increase in the concentration of calcium ion in the growth medium can cause the keratinocytes within the matrix to stratify in a manner similar to what occurs in the skin (Bell et al., J. Biomech. Eng. 113:113-119, 1991; Parenteau et al., J. Cell. Biochem. 45:245-251, 1991). Once differentiated, the keratinocytes lay down basal lamina consisting of collagen, glycosaminoglycans, and laminin. These basal lamina components as well as extracellular matrix proteins, which can be either synthesized by fibroblasts or included in the production medium, provide a physiological framework that is advantageous to fibroblast survival after formation of matrices from the mixtures introduced into a subject.

**EXAMPLE XXI**

Endothelial cells form the lumen of blood vessels and capillaries. Vessels are formed from single endothelial cells that divide and differentiate to form contiguous tubes. This differentiation process can be stimulated in the presence of essential environmental factors. These factors include collagen, extracellular matrix proteins produced by cells such as fibroblasts and/or keratinocytes, and growth factors (e.g., basic fibroblast growth factor or vascular endothelial growth factor). Any of the matrices generated from the mixtures described herein can provide the proper environment for endothelial tube formation if endothelial cells are added to the mixtures along with fibroblasts. The addition of keratinocytes can help induce differentiation of the endothelial cells through secretion of factors such as vascular endothelial growth factor.
as well as production of basal lamina. Endothelial cells can also be beneficial by releasing factors that promote neovascularization.

**EXAMPLE XXII**

5 The mixtures of the invention would be prepared for introduction into humans as follows:

The desired cells, typically stably transfected autologous cells derived from the patient, are harvested from tissue culture dishes and processed for the production of HCMM or PCHCMM by any of the methods described above. The dosage for a given patient (i.e., the physiologically effective quantity of therapeutic product produced by the matrix) can be varied by introducing a larger or smaller volume of the mixture into the patient, and/or using cells which express a different level of the product per cell when making the mixture. The quantity of the therapeutic product produced in the patient may also be varied by exposing the cells in the mixture to a pharmacologic or physiologic signal which alters expression of the therapeutic gene. For example, if the therapeutic gene is under the control of a glucocorticoid-responsive promoter, then *in vivo* exposure of the cells to a drug such as dexamethasone (by administering the drug to the patient in a manner that ensures the drug reaches the implant) will alter expression of the therapeutic gene. Other pharmacologically responsive control elements are described in Clackson (2000), *Gene Therapy* 7:120-125 which is incorporated herein by reference in its entirety. Such elements include those regulated by the antibiotic tetracycline, or its analogs (e.g., doxycycline), the insect steroid ecdysone or its analogs, the antiprogestin mifepristone (RU486), and chemical "dimerizers" such as rapamycin and its analogs ("rapalogs").

25 The mixtures can contain any of the microcarriers, collagens (or alternatives), agents (optionally attached to solid substrates), and cells producing agents described herein. They can also optionally contain keratinocytes and/or endothelial cells. Microcarriers filled with any of the cells recited herein can be frozen and then thawed prior to use in the injectable mixtures.
The mixture can be introduced at a variety of sites, including but not limited to subcutaneous, intraperitoneal, intrasplenic, intraomental, inguinal, intrathecal, intraventricular, and intramuscular sites, as well as within lymph nodes or within adipose tissue. The mixture can be introduced using, for example, a hypodermic syringe (plastic or glass) optionally capped with a needle or catheter. The diameter of the needle or catheter is preferably larger than the diameter of microcarriers (e.g., greater than 0.5 mm) to minimize damage to the suspended cells and microcarriers. Delivery to an appropriate internal site can be facilitated by the use of a laparoscope. Under certain circumstances, it may be desirable to make a surgical incision, introduce the mixture and then close the incision.

Furthermore, the mixtures of the invention can be simultaneously formed and introduced into a patient (or any other animal). An appropriate apparatus can be constructed for this purpose. An example of such an apparatus is shown in Fig. 15A. The apparatus can contain two or more vessels 1, all of which connect via outlets 2 to inlets 3 in an adapter piece 4. Figure 15B shows a cross-sectional view of such an adapter piece 4. The inlets 3 in the adapter piece 4 are in fluid communication, preferably in a liquid-tight fashion, with the outlets 2 from the two or more vessels. Alternatively, the adapter piece 4 can contain a single connector that is in fluid communication, preferably in a liquid-tight fashion, with all the outlets 2 of the vessels 1. The adapter piece also contains a mixing chamber 5 and an outlet 6 that is in fluid communication, preferably in a liquid-tight fashion, with the single inlet in the hub 7 of a syringe needle 8. In this way, when the contents of the vessels are caused (by simultaneously applying pressure to pistons 12) to flow from the outlets 2 of the vessels 1, they enter the mixing chamber 5 in the adapter piece 4 where they mix, and the resulting mixture flows out of the outlet 5 in the adapter piece 4 and into the syringe needle 8. The mixture is then forced into the needle portion 13 of the syringe needle and, hence, into a tissue or body space of an animal into which the needle has been inserted. The vessels are physically joined to each other by solid connectors 9. A "pressure plate" 10 connecting the tops of pistons 12 is included in order to facilitate simultaneous extrusion of the contents of the vessels.
In another embodiment, instead of an adapter piece, the hub section of the syringe needle can be adapted to have separate inlets that are in fluid communication, preferably in a liquid-tight manner, with the individual outlets in the vessels. When the contents of all the vessels are caused to flow out of the outlets of the vessels (e.g., by simultaneously applying pressure to the plungers, where the vessels are syringes), they enter the hub of the syringe needle via the appropriate inlets in the hub and mix in the small chamber formed within the hub before entering the needle portion of the apparatus. As used herein, a "delivery means" can be: (a) a syringe needle adapted as described, (b) the above described adapter piece 4 and the syringe needle 8, (c) a catheter, (d) a cannula, (e) a pipette or any tube-shaped object, appropriately adapted, that will function as described.

In an apparatus with two vessels, one of the vessels can contain, for example, cells, microcarriers, and agents in a nutrient medium, and the other can contain a collagen solution. In a three-vessel apparatus, one vessel can contain, for example, cells, microcarriers, and agents in nutrient medium, the second vessel can contain a collagen solution at an acid pH, and the third can contain medium or diluent at an alkaline pH, such that when the contents of all three vessels mix, an aqueous suspension at a pH of 7.2-7.4 is formed. It is understood, however, that the various components of the hybrid matrix mixtures of the invention can be distributed among the two or three vessels of the device in any convenient combination. While in using such an apparatus, introduction of the mixture into the animal occurs shortly after formation of the mixture from its components, for the purposes of the invention, the two events can be said to occur simultaneously.

The invention includes a vessel containing any of the mixtures disclosed herein (see "Summary of the Invention"). Such a vessel can be included together with shipping material (e.g., a shipping container), in a kit. The feasibility of shipping the injectable mixtures in a single vessel containing all of the components was evaluated. Two studies with injectable HSHCM were performed. For both, the heparin-Sepharose bead component was coated with bFGF at a concentration of 50 μg/mL packed beads. A volume of 10mL of complete production medium, prepared as
outlined in Table 14, was added to each of three 10cc plastic syringes, which were then capped and stored at 4°C for 24h. The syringes were removed from the 4°C environment, capped with 16G needles, and the contents injected into 100mm petri dishes. The dishes were placed in an incubator at a temperature of 37°C, humidity of 95% to 98%, and CO₂ level of 5%. After 1h, the dishes were removed and fed by addition of 20mL of DMEM without phenol red, and returned to the incubator. The decrease in diameter of the solidified matrix, relative to the diameter of the liquid mixture ("reduction in diameter"), cell number, and cell viability were assessed after 24h of incubation at 37°C. The "reduction in diameter" was determined by placing a ruler beneath each dish, measuring the matrix diameter in centimeters, and subtracting the matrix diameter from the diameter of the 100mm dish. The difference in diameter was then divided by the diameter of the 100mm dish and expressed as a percentage. Cell number and viability were assessed as described above for HCM. Table 15 summarizes the results of the two storage experiments. Experiments #1 and #2 differed in the number of cells incorporated per mL production medium as described (n=3 syringes per study).

<table>
<thead>
<tr>
<th>Exp#</th>
<th>#Cells/10mL (x 10⁶)</th>
<th>Time of 4°C Storage</th>
<th>Time of 37°C Incubation</th>
<th># Cells Recovered (x 10⁶)</th>
<th>% Viability</th>
<th>% Reduction in Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44.7</td>
<td>24h</td>
<td>24h</td>
<td>39.5+/−4.1</td>
<td>92+/−1.5</td>
<td>26.9+/−3.9</td>
</tr>
<tr>
<td>2</td>
<td>91.0</td>
<td>24h</td>
<td>24h</td>
<td>93.4+/−5.1</td>
<td>94+/−3.7</td>
<td>35.4+/−0.7</td>
</tr>
</tbody>
</table>

Alternatively, since a neutralized collagen solution will gel more rapidly than an acidic collagen solution, it could be advantageous to separate the components of the injectable material in a kit for the purpose of shipping the material to an offsite location. For example, a kit could contain three vessels (e.g., tubes, syringes, bottles, or vials), the contents of which are combined immediately prior to introduction into
the patient (i.e., before the mixture has solidified to such an extent that it does not flow readily from the syringe, cannula, or whatever means is used to deliver the suspension). The components could, for example, be separated in the following manner: vessel 1 can contain cells + microcarriers, and, optionally, heparin-Sepharose beads in a slurry; vessel 2 can contain a collagen solution at acidic pH (about 2.0 - about 4.0); and vessel 3 can contain concentrated medium (e.g., nutrient medium or other diluent) without serum and at alkaline pH (about 9.0 - about 12.0). The pH of the concentrated medium would be selected to ensure neutralization of the collagen solution to a physiological pH (e.g., pH 7.4) when the medium and collagen solutions are combined. Immediately prior to injection into the patient, the contents of vessel 3 (medium) would be mixed with the contents of vessel 2 (collagen solution). The combined medium + collagen, which will at this time be neutralized to physiological pH, would then be added to the cell/microcarrier/heparin-Sepharose bead slurry and mixed thoroughly to form the final mixture. This final mixture would then be injected into the patient, optionally using a hypodermic syringe or other liquid-dispensing apparatus supplied with the kit.

Alternatively, a kit could contain two vessels, with the first vessel containing the same components as vessel 1 in the kit described above. The second vessel could contain a collagen solution in which the collagen is dissolved, for example, in nutrient medium and which is at an acidic pH. Immediately prior to injection, the pH of the collagen solution is adjusted to about 7.2-7.4 using an alkaline solution (e.g., a NaOH solution). An aliquot of such an alkaline solution could optionally be included in a third vessel in the kit.

Instead of providing the collagen solution at an acidic pH, it can be provided at a pH of 7.2-7.4, as long as it is kept cold (e.g., below 8°C) until immediately prior to mixing with the other components and introduction into the patient.

The kits of the invention can optionally include labels or inserts with instructions for use of the kit, e.g., instructions on how to combine the contents of the vessels and administer the resulting mixture to a subject. Keeping the mixture cold prevents gelling of the collagen.
Other Embodiments

The injectable mixtures of the invention are appropriate for delivery of a wide range of cellular products, including not only hGH and Factor VIII, but also Factor IX, erythropoietin (EPO), albumin, hemoglobin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, low density lipoprotein (LDL) receptor, IL-2 receptor, globin, immunoglobulin, catalytic antibodies, the interleukins, insulin, insulin-like growth factor 1 (IGF-1), insulinotropin, parathyroid hormone (PTH), leptin, an IFN (e.g., IFN-α, IFN-β, or IFN-ω), the nerve growth factors, basic fibroblast growth factor (bFGF), acidic FGF (aFGF), epidermal growth factor (EGF), endothelial cell growth factor, platelet derived growth factor (PDGF), transforming growth factors, endothelial cell stimulating angiogenesis factor (ESAF), angiogenin, tissue plasminogen activator (t-PA), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), α-galactosidase, and FSH. For example, the cells in the mixtures can be pancreatic beta cells which naturally secrete insulin in response to a rise in blood glucose, and which therefore can supplement an inadequate insulin response in a diabetic or pre-diabetic patient. Alternatively, they can be any type of cell genetically engineered with a DNA construct to express and secrete high levels of a needed polypeptide, such as a clotting factor, within the patient. Such a DNA construct can contain a protein-encoding sequence under the control of a constitutively activated promoter, or of an appropriately physiologically or pharmacologically regulated promoter.

The collagen portion of the injectable CMM can consist entirely of collagen, or can contain other components instead of or in addition to collagen: e.g., agarose; alginate; a glycosaminoglycan such as hyaluronic acid, heparin sulfate, dermatan sulfate, or chondroitin sulfate; a sulfated proteoglycan; fibronectin; laminin; elastin; fibrin; tenascin; minced adipose tissue; minced omental tissue; methyl cellulose; or gelatin. Such components (particularly those which are found in the extracellular matrix of animal tissues) contribute to the structural stability of the hybrid matrices once solidified in the animal, and/or provide additional attachment capacity for the cells in the matrices.
and the host tissue at the site of introduction. They would be mixed into the matrix mixture prior to introduction into an animal.

While the mixtures of the invention will preferably contain collagen (or one or more of the alternatives listed above), it is understood that the invention also includes mixtures lacking collagen (or any of the above alternatives). In such a case (i.e., when no matrix material is used), the injected solution does not solidify to form a discrete mass within the animal. The degree to which the injected material disperses from the site of implantation depends on the structural characteristics of the site. Dispersion of the microcarrier-bound cells may be preferred in some situations.

Other potential additives include cytokines and/or growth factors which are useful for optimizing maintenance of the cells or promoting beneficial interaction with host tissue (e.g., vascularization), including bFGF, aFGF, endothelial cell growth factor, PDGF, endothelial cell stimulating angiogenesis factor (ESAF), an angiopoietin, leukotriene C₄, prostaglandins (e.g., PGE₁, PGE₂), IGF-1, G-CSF, angiogenin, TGF-α, TGF-β, ascorbic acid, EGF, oncostatin M, VEGF, VEGF-A, VEGF-B, VEGF-C, and VEGF-D. These additives can, for example, be incorporated into the matrix by combining them in the injectable mixtures prior to introduction into an animal, or by introducing them into the interstices of the microcarriers. They can also be bound to or encapsulated within separate solid substrates (e.g., heparin-coated agarose beads or PLGA microcapsules) included in the mixtures. Alternatively, the cells may be genetically engineered to express the desired product. For example, the cells may be cotransfected with a DNA encoding an angiogenesis factor and a DNA encoding a second therapeutic protein, or with a single DNA construct encoding both types of proteins linked to suitable expression control sequences.

Ascorbic acid promotes the production of mature collagen by fibroblasts by promoting the proper post-translational modification of procollagen. The production of collagen by fibroblasts included in the injectable CMM will increase the physical strength of the matrix produced from the injectable CMM as well as provide a physiological architecture that may increase cell survival in an animal.
The collagen used in the gel may be any suitable type (e.g., type I-XI), or a mixture of any two or more. If fibers are added, they would be made principally of collagen (e.g., cat gut) or a non-collagenous material such as nylon, dacron, polytetrafluoroethylene (Gore-Tex™ or Teflon™), polyglycolic acid, polylactic/polyglycolic acid mixture (Vicryl™), polystyrene, polyvinylchloride copolymer, cellulose (e.g., cotton or linen), polyester, rayon, or silk.

Instead of the type I collagen microcarriers described in the above examples, one could utilize microcarriers consisting primarily of another type of collagen, polystyrene, dextran (e.g., Cytodex™, Pharmacia), polyacrylamide, cellulose, calcium alginate, latex, polysulfone, glass (coated with a substance such as collagen which promotes cellular adherence), gelatin, or combinations of collagen with any of the above. Such microcarriers are available commercially or can be made by standard methods, then sterilized for use in the mixtures of the invention.

Other embodiments are within the following claims.
What is claimed is:

1. A method of administering a polypeptide to a patient in need thereof, the method comprising
   providing a fluid mixture comprising, suspended in aqueous solution,
   a population of cultured vertebrate cells that express the
   polypeptide, and
   a plurality of microcarriers; and
   introducing the fluid mixture into the patient.

2. The method of claim 1, wherein the cultured vertebrate cells are
   genetically engineered to express the polypeptide.

3. The method of claim 1, the mixture further comprising soluble collagen.

4. The method of claim 1, the mixture further comprising a substance selected from the group consisting of minced adipose tissue, minced omental tissue, methyl cellulose, alginate, gelatin, and fibrin.

5. The method of claim 3, wherein the collagen is type I.

6. The method of claim 3, wherein the collagen is type III.

7. The method of claim 3, wherein the collagen is type IV.

8. The method of claim 1, wherein the cultured vertebrate cells are derived from one or more cells removed from the patient, and have been genetically engineered \textit{in vitro} to express and secrete the polypeptide.

9. The method of claim 1, wherein the introduction is at a subcutaneous site in the patient.

10. The method of claim 1, wherein the introduction is at an intraperitoneal, sub-renal capsular, inguinal, intramuscular, intraventricular, intraomental, or intrathecal site in the patient.

11. The method of claim 1, wherein the polypeptide is one which promotes wound healing, and the introduction is at a site of a preexisting wound of the patient.
12. The method of claim 1, wherein each of the plurality of microcarriers consists primarily of collagen.

13. The method of claim 12, wherein the microcarriers are porous.

14. The method of claim 1, wherein each of the plurality of microcarriers consists primarily of gelatin.

15. The method of claim 14, wherein the microcarriers are porous.

16. The method of claim 1, wherein each of the plurality of microcarriers consists primarily of one or more substances selected from the group consisting of polystyrene, dextran, polyacrylamide, cellulose, calcium alginate, latex, polysulfone, and glass.

17. The method of claim 1, wherein the cultured vertebrate cells are selected from the group consisting of adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, and precursors of any of the above.

18. The method of claim 1, wherein the cultured vertebrate cells are human cells.

19. The method of claim 1, wherein the polypeptide is selected from the group consisting of enzymes, hormones, cytokines, colony stimulating factors, vaccine antigens, antibodies, clotting factors, regulatory proteins, transcription factors, receptors, and structural proteins.

20. The method of claim 1, wherein the polypeptide is Factor VIII.

21. The method of claim 1, wherein the polypeptide is human growth hormone.

22. The method of claim 1, wherein the polypeptide is Factor IX.

23. The method of claim 1, wherein the polypeptide is erythropoietin.
24. The method of claim 1, wherein the polypeptide is selected from the group consisting of VEGF, VEGF-A, VEGF-B, VEGF-C, and VEGF-D.

25. The method of claim 1, wherein the polypeptide is insulinotropin.

26. The method of claim 1, wherein the polypeptide is angiopoietin 1, 2, 3, or 4.

27. The method of claim 1, wherein the polypeptide is selected from the group consisting of alpha-1 antitrypsin, calcitonin, glucocerebrosidase, low density lipoprotein (LDL) receptor, IL-2 receptor, globin, immunoglobulin, catalytic antibodies, the interleukins, insulin, insulin-like growth factor 1 (IGF-1), parathyroid hormone (PTH), leptin, the nerve growth factors, basic fibroblast growth factor (bFGF), acidic FGF (aFGF), epidermal growth factor (EGF), endothelial cell growth factor, platelet derived growth factor (PDGF), transforming growth factors, endothelial cell stimulating angiogenesis factor (ESAF), angiogenin, tissue plasminogen activator (t-PA), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF).

28. The method of claim 1, wherein the polypeptide is selected from the group consisting of interferon-α (IFN-α), interferon-β (IFN-β), interferon-γ (IFN-γ), follicle stimulating hormone (FSH), α-galactosidase, β-glucuronidase, α-iduronidase, α-L-iduronidase, glucosamine-N-sulfatase, α-N-acetylgulosaminidase, acetylcoenzyme A:α-glucosaminide-N-acetyltransferase, N-acetylgulosamine-6-sulfatase, β-galactosidase, N-acetylgalactosamine-6-sulfatase, and β-glucuronidase.

29. The method of claim 1, wherein the microcarriers are beads of type I collagen.

30. The method of claim 29, wherein the microcarriers are porous.

31. The method of claim 1, wherein the microcarriers are beads of porous gelatin.

32. The method of claim 1, wherein the majority of the microcarriers have an approximately spherical shape and have a diameter between approximately 0.1 and approximately 2 mm.
33. The method of claim 2, wherein the mixture additionally comprises a substance selected from the group consisting of a second type of collagen, agarose, alginate, fibronectin, laminin, hyaluronic acid, heparan sulfate, dermatan sulfate, sulfated proteoglycans, fibrin, elastin, tenascin, heparin, cellulose, starch, dextran, chitosan, minced adipose tissue, minced omental tissue, methyl cellulose, and gelatin.

34. The method of claim 2, wherein the mixture additionally comprises noncollagen fibers.

35. The method of claim 34, wherein the noncollagen fibers comprise a material selected from the group consisting of nylon, dacron, polytetrafluoroethylene, polyglycolic acid, polylactic/polyglycolic acid copolymer, polystyrene, polyvinylchloride, cotton, linen, polyester, and silk.

36. The method of claim 1, wherein the cultured vertebrate cells are a clonal population derived from one or more cells removed from the patient.

37. The method of claim 1, wherein the cultured vertebrate cells are fibroblasts.

38. The method of claim 1, wherein the mixture further comprises an agent selected from the group consisting of a factor which promotes vascularization, a cytokine, a growth factor, and ascorbic acid.

39. The method of claim 38, wherein the mixture further comprises a solid substrate associated with the agent.

40. The method of claim 39, wherein the solid substrate comprises heparin or heparan sulfate proteoglycan.

41. The method of claim 39, wherein the solid substrate comprises agarose with heparin or heparan sulfate proteoglycan bound thereto.

42. The method of claim 41, wherein the solid substrate further comprises calcium alginate.

43. The method of claim 39, wherein the solid substrate further comprises calcium alginate.
44. The method of claim 39, wherein the solid substrate comprises a substance selected from the group consisting of collagen, gelatin, ethylene-vinyl acetate, polylactide/glycolic acid co-polymer, fibrin, sucrose octasulfate, dextran, polyethylene glycol, an alginate, polyacrylamide, cellulose, latex, and polyhydroxyethylmethacrylate.

45. The method of claim 44, wherein heparin or heparan sulfate proteoglycan is bound to the substance.

46. The method of claim 38, wherein the mixture further comprises a second agent selected from the group consisting of a factor which promotes vascularization, a cytokine, and a growth factor.

47. The method of claim 39, wherein the solid substrate is in the form of beads.

48. The method of claim 39, wherein the solid substrate is in the form of threads.

49. The method of claim 38, wherein the agent is basic fibroblast growth factor (bFGF).

50. The method of claim 38, wherein the agent is selected from the group consisting of acidic fibroblast growth factor (aFGF), endothelial cell growth factor, platelet-derived growth factor (PDGF), endothelial cell stimulating angiogenesis factor (ESAF), leukotriene C4, a prostaglandin, insulin-like growth factor 1 (IGF-1), granulocyte colony stimulating factor (G-CSF), angiogenin, transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), ascorbic acid, epidermal growth factor (EGF), and oncostatin M.

51. The method of claim 38, wherein the agent is selected from the group consisting of vascular endothelial growth factor (VEGF), VEGF-A, VEGF-B, VEGF-C, and VEGF-D.

52. The method of claim 38, wherein the agent is angiopoietin 1, 2, 3, or 4.
53. The method of claim 36, wherein the mixture comprises cells secreting
the agent.

54. The method of claim 39, wherein the solid substrate and the plurality
of microcarriers are the same entities.

55. The method of claim 1, wherein the mixture further comprises a
second population of cultured vertebrate cells secreting an agent selected from the
group consisting of a factor which promotes vascularization, a cytokine, and a growth
factor.

56. The method of claim 55, wherein the cells of the second population of
cultured vertebrate cells are genetically engineered to express the agent.

57. The method of claim 55, wherein the first and second populations are
the same population of cells.

58. The method of claim 57, wherein the cultured vertebrate cells are
cotransfected with a DNA encoding the polypeptide and a DNA encoding the agent.

59. The method of claim 57, wherein the cultured vertebrate cells are
transfected with a single DNA encoding both the polypeptide and the agent.

60. The method of claim 55, wherein the mixture further comprises a third
population of cultured vertebrate cells which express and secrete a second agent
selected from the group consisting of a factor that promotes vascularization, a
cytokine, and a growth factor.

61. The method of claim 55, wherein the agent is bFGF.

62. The method of claim 55, wherein the agent is selected from the group
consisting of VEGF, VEGF-A, VEGF-B, VEGF-C, and VEGF-D.

63. The method of claim 55, wherein the agent is angiopoietin 1, 2, 3, or 4.

64. The method of claim 55, wherein the agent is selected from the group
consisting of aFGF, endothelial cell growth factor, PDGF, ESAF, leukotriene C4, a
prostaglandin, IGF-1, G-CSF, angiogenin, TGF-α, TGF-β, EGF, and oncostatin M.
65. The method of claim 55, wherein the second population of cultured vertebrate cells is selected from the group consisting of adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, and precursors of any of the above.

66. The method of claim 55, wherein the cultured vertebrate cells of the first and second populations are human cells.

67. The method of claim 37, wherein the mixture further comprises keratinocytes.

68. The method of claim 67, wherein the keratinocytes and the fibroblasts are obtained from the same individual.

69. The method of claim 67, further comprising a keratinocyte differentiation factor.

70. The method of claim 69, wherein the keratinocyte differentiation factor comprises calcium ions at a concentration of 1.5-2 mM.

71. The method of claim 1, wherein the mixture further comprises endothelial cells.

72. The method of claim 71, wherein the cultured vertebrate cells and the endothelial cells are obtained from the same individual.

73. The method of claim 1, wherein the introduction of the mixture into the patient is by a hypodermic syringe.

74. The method of claim 73, wherein the syringe is capped with a syringe needle or a catheter.

75. The method of claim 1, wherein the introduction into the patient is by a catheter.

76. The method of claim 1, wherein the introduction into the patient is by a pipette.
77. The method of claim 2, wherein combining the cultured vertebrate cells, the microcarriers and the soluble collagen to form the mixture occurs simultaneously with introducing the mixture into the patient.

78. A method of administering a polypeptide to a patient in need thereof, the method comprising

providing a fluid mixture comprising an aqueous solution of collagen and, suspended in the solution,

a population of cultured vertebrate cells that express the polypeptide,

and

a plurality of microcarriers; and

introducing the fluid mixture into the patient.

79. The method of claim 78, wherein the cultured vertebrate cells are genetically engineered to express the polypeptide.

80. A kit comprising a shipping container containing

(a) a first vessel comprising

(i) a population of cultured vertebrate cells genetically engineered to express a polypeptide, and

(ii) a plurality of microcarriers;

(b) a second vessel comprising a collagen solution; and

(c) a third vessel comprising medium.

81. The kit of claim 80, wherein the cultured vertebrate cells are genetically engineered to express the polypeptide.

82. The kit of claim 80, wherein the collagen solution is at an acidic pH and the medium is at an alkaline pH.

83. The kit of claim 80, wherein the collagen solution is at a temperature below 8°C.

84. The kit of claim 80, wherein the collagen is type I.
85. The kit of claim 80, wherein the collagen is type III.
86. The kit of claim 80, wherein the collagen is type IV.
87. The kit of claim 80, wherein the cultured vertebrate cells are derived from one or more cells removed from the patient, and have been genetically engineered in vitro to express and secrete the polypeptide.

88. The kit of claim 80, wherein the polypeptide is one which promotes wound healing, and the introduction is at a site of a preexisting wound of the patient.
89. The kit of claim 80, wherein each of the plurality of microcarriers consists primarily of collagen.

90. The kit of claim 80, wherein each of the plurality of microcarriers consists primarily of gelatin.
91. The kit of claim 90, wherein the microcarriers are porous.
92. The kit of claim 80, wherein each of the plurality of microcarriers consists primarily of one or more substances selected from the group consisting of polystyrene, dextran, polyacrylamide, cellulose, calcium alginate, latex, polysulfone, and glass.

93. The kit of claim 80, wherein the cultured vertebrate cells are selected from the group consisting of adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, and precursors of any of the above.

94. The kit of claim 80, wherein the cultured vertebrate cells are human cells.

95. The kit of claim 80, wherein the polypeptide is selected from the group consisting of enzymes, hormones, cytokines, colony stimulating factors, vaccine antigens, antibodies, clotting factors, regulatory proteins, transcription factors, receptors, and structural proteins.
96. The kit of claim 80, wherein the polypeptide is Factor VIII.

97. The kit of claim 80, wherein the polypeptide is human growth hormone.

98. The kit of claim 80, wherein the polypeptide is Factor IX.

99. The kit of claim 80, wherein the polypeptide is erythropoietin.

100. The kit of claim 80, wherein the polypeptide is selected from the group consisting of VEGF, VEGF-A, VEGF-B, VEGF-C, and VEGF-D.

101. The kit of claim 80, wherein the polypeptide is angiopoietin 1, 2, 3, or 4.

102. The kit of claim 80, wherein the polypeptide is insulinotropin.

103. The kit of claim 80, wherein the polypeptide is selected from the group consisting of alpha-1 antitrypsin, calcitonin, glucocerebrosidase, low density lipoprotein (LDL) receptor, IL-2 receptor, globin, immunoglobulin, catalytic antibodies, the interleukins, insulin, insulin-like growth factor 1 (IGF-1), parathyroid hormone (PTH), leptin, the nerve growth factors, basic fibroblast growth factor (bFGF), acidic FGF (aFGF), epidermal growth factor (EGF), endothelial cell growth factor, platelet derived growth factor (PDGF), transforming growth factors, endothelial cell stimulating angiogenesis factor (ESAF), angiogenin, tissue plasminogen activator (t-PA), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

104. The kit of claim 77, wherein the polypeptide is selected from the group consisting of IFN-α, IFN-β, IFN-γ, FSH, α-galactosidase, β-glucuronidase, α-iduronidase, α-L-iduronidase, glucosamine-N-sulfatase, α-N-acetylg glucosaminidase, acetylcoenzyme A:α-glucosaminide-N-acetyltransferase, N-acetylg glucosaminide-6-sulfatase, β-galactosidase, N-acetylglactosamine-6-sulfatase, and β-glucuronidase.

105. The kit of claim 80, wherein the microcarriers are beads of type I collagen.

106. The kit of claim 105, wherein the microcarriers are porous.
107. The kit of claim 80, wherein the microcarriers are beads of porous gelatin.

108. The kit of claim 80, wherein the majority of the microcarriers have an approximately spherical shape and have a diameter between approximately 0.1 and approximately 2 mm.

109. The kit of claim 80, wherein the first or the second vessel additionally comprises a substance selected from the group consisting of a second type of collagen, agarose, alginate, fibronectin, laminin, hyaluronic acid, heparan sulfate, dermatan sulfate, sulfated proteoglycans, fibrin, elastin, tenascin, heparin, cellulose, starch, dextran, chitosan, minced adipose tissue, minced omental tissue, methyl cellulose, and gelatin.

110. The kit of claim 80, wherein the first or the second vessel additionally comprises noncollagen fibers.

111. The kit of claim 110, wherein the noncollagen fibers comprise a material selected from the group consisting of nylon, dacron, polytetrafluoroethylene, polyglycolic acid, polylactic/polyglycolic acid copolymer, polystyrene, polyvinylchloride, cotton, linen, polyester, and silk.

112. The kit of claim 80, wherein the cultured vertebrate cells are a clonal population derived from one or more cells removed from a patient.

113. The kit of claim 80, wherein the cultured vertebrate cells are fibroblasts.

114. The kit of claim 80, the first vessel further comprising an agent selected from the group consisting of a factor that promotes vascularization, a cytokine, a growth factor, and ascorbic acid.

115. The kit of claim 114, wherein the first vessel further comprises a solid substrate associated with the agent.

116. The kit of claim 115, wherein the solid substrate comprises heparin or heparan sulfate proteoglycan.
117. The kit of claim 115, wherein the solid substrate comprises agarose with heparin or heparan sulfate proteoglycan bound thereto.

118. The kit of claim 117, wherein the solid substrate further comprises calcium alginate.

119. The kit of claim 115, wherein the solid substrate further comprises calcium alginate.

120. The kit of claim 115, wherein the solid substrate comprises a substance selected from the group consisting of collagen, gelatin, ethylene-vinyl acetate, polylactide/glycolic acid co-polymer, fibrin, sucrose octasulfate, dextran, polyethylene glycol, an alginate, polyacrylamide, cellulose, latex, and polyhydroxyethylmethacrylate.

121. The kit of claim 120, wherein heparin or heparan sulfate proteoglycan is bound to the substance.

122. The kit of claim 114, wherein the first vessel further comprises a second agent selected from the group consisting of a factor which promotes vascularization, a cytokine, and a growth factor.

123. The kit of claim 115, wherein the solid substrate is in the form of beads.

124. The kit of claim 115, wherein the solid substrate is in the form of threads.

125. The kit of claim 114, wherein the agent is basic fibroblast growth factor (bFGF).

126. The kit of claim 114, wherein the agent is selected from the group consisting of acidic fibroblast growth factor (aFGF), endothelial cell growth factor, platelet-derived growth factor (PDGF), endothelial cell stimulating angiogenesis factor (ESAF), leukotriene C₄, a prostaglandin, insulin-like growth factor 1 (IGF-1), granulocyte colony stimulating factor (G-CSF), angiogenin, transforming growth
factor-α (TGF-α), transforming growth factor-β (TGF-β), ascorbic acid, epidermal
growth factor (EGF), and oncostatin M.

127. The kit of claim 114, wherein the agent is selected from the group
consisting of vascular endothelial growth factor (VEGF), VEGF-A, VEGF-B, VEGF-
C, and VEGF-D.

128. The kit of claim 114, wherein the agent is angiopoietin 1, 2, 3, or 4.

129. The kit of claim 114, wherein the first vessel further comprises cells
secrating the agent.

130. The kit of claim 115, wherein the solid substrate and the plurality of
microcarriers are the same entities.

131. The kit of claim 80, the first vessel further comprising a second
population of cultured vertebrate cells secreting an agent selected from the group
consisting of a factor that promotes vascularization, a cytokine, and a growth factor.

132. The kit of claim 131, wherein the cells of the second population of
cultured vertebrate cells are genetically engineered to express the agent.

133. The kit of claim 131, wherein the first and second populations are the
same population of cells.

134. The kit of claim 133, wherein the cultured vertebrate cells are
cotransfected with a DNA encoding the polypeptide and a DNA encoding the agent.

135. The kit of claim 133, wherein the cultured vertebrate cells are
transfected with a single DNA encoding both the polypeptide and the agent.

136. The kit of claim 131, wherein the first vessel further comprises a third
population of cultured vertebrate cells which express and secrete a second agent
selected from the group consisting of a factor that promotes vascularization, a
cytokine, and a growth factor.

137. The kit of claim 131, wherein the agent is bFGF.

138. The kit of claim 131, wherein the agent is selected from the group
consisting of VEGF, VEGF-A, VEGF-B, VEGF-C, and VEGF-D.
139. The kit of claim 131, wherein the agent is angiopoietin 1, 2, 3, or 4.

140. The kit of claim 131, wherein the agent is selected from the group consisting of aFGF, endothelial cell growth factor, PDGF, ESAF, leukotriene C4, a prostaglandin, IGF-1, G-CSF, angiogenin, TGF-α, TGF-β, ascorbic acid, EGF, and oncostatin M.

141. The kit of claim 131, wherein the second population of cultured vertebrate cells is selected from the group consisting of adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, and precursors of any of the above.

142. The kit of claim 131, wherein the cultured vertebrate cells of the first and second populations are human cells.

143. The kit of claim 80, wherein the cultured vertebrate cells are fibroblasts.

144. The kit of claim 143, wherein the first vessel further comprises keratinocytes.

145. The kit of claim 144, wherein the keratinocytes and the fibroblasts are obtained from the same individual.

146. The kit of claim 144, further comprising a keratinocyte differentiation factor.

147. The kit of claim 146, wherein the keratinocyte differentiation factor comprises calcium ions at a concentration of 1.5-2 mM.

148. The kit of claim 80, wherein the first vessel further comprises endothelial cells.

149. A method of preparing a mixture using the kit of claim 80, the method comprising:

(a) combining the contents of the second and third vessels to form a diluted
collagen solution; and

(b) combining the diluted collagen solution with the contents of the first vessel to form a mixture.

150. The method of claim 149, further comprising the step of introducing the mixture into a patient prior to solidification of the mixture.

151. A kit comprising a shipping container containing

(a) a first vessel comprising

(i) a population of cultured vertebrate cells that express a polypeptide, and

(ii) a plurality of microcarriers; and

(b) a second vessel comprising a collagen solution.

152. The kit of claim 151, wherein the cultured vertebrate cells are genetically engineered to express the polypeptide.

153. The kit of claim 151, wherein the collagen solution is at an acidic pH.

154. The kit of claim 151, wherein the collagen solution is at a temperature below about 8°C.

155. The kit of claim 150, further comprising a third vessel comprising a solution at an alkaline pH.

156. A method of preparing a mixture using the kit of claim 151, the method comprising combining the contents of the first and second vessels to form a mixture.

157. The method of claim 156, further comprising the step of introducing the mixture into a patient prior to solidification of the mixture.

158. A kit comprising a shipping container containing a vessel comprising an aqueous solution of collagen and, suspended in the solution,

(a) a population of cultured vertebrate cells that express a polypeptide; and
(b) a plurality of microcarriers, wherein the aqueous solution is at a
temperature below about 8°C.

159. The kit of claim 158, wherein the cultured vertebrate cells are
genetically engineered to express the polypeptide.

160. A method of administering a polypeptide to a patient in need thereof,
the method comprising:

providing a fluid collagen mixture comprising, suspended in an aqueous
collagen solution, a population of cultured vertebrate cells that express the
polypeptide; and

10 introducing the fluid mixture into the patient.

161. The method of claim 160, wherein the cultured vertebrate cells are
genetically engineered to express the polypeptide.

162. An apparatus for introduction of a mixture into a patient, the apparatus
comprising at least two vessels, each of the vessels having an outlet in fluid
communication with a delivery means, wherein the apparatus comprises (a) an
aqueous collagen solution, (b) a population of cultured vertebrate cells that express a
polypeptide, and (c) a plurality of microcarriers.

163. The apparatus of claim 162, wherein the cultured vertebrate cells are
genetically engineered to express the polypeptide.

164. The apparatus of claim 162, wherein one or more of (a), (b), or (c) are
contained within each vessel.

165. The apparatus of claim 162, wherein one or more of the vessels further
has associated therewith a piston having two ends, a first end adapted to be inserted
into the interior of the vessel, and a second end adapted to extend from the interior of
the vessel.

166. The apparatus of claim 162, wherein the delivery means is a syringe
needle having (a) one or more inlets adapted to be in fluid communication with the
outlets of the vessels, and (b) an outlet.
167. The apparatus of claim 162, wherein the delivery means comprises an adapter piece having a connector in fluid connection with the outlet of each of the vessels, a mixing chamber, and an outlet in fluid communication with a syringe needle.

168. The apparatus of claim 167, wherein the connector comprises at least two inlets, each in fluid communication with the outlet of only one of the vessels.

169. The apparatus of claim 162, further comprising one or more connections physically joining the at least two vessels.

170. The apparatus of claim 165, wherein the second end of each piston is connected to a pressure plate.
FIG. 2
FIG. 3

- TATA (13331)
- CAP (1)
- SD (285)
- SA (1117)
- SD (1131)
- SA (2074)
- ATG (2080)
- CMV enhancer
- fibronectin Promoter
- \(\beta\)-actin 5' intron
- \(E\)F1\(\alpha\)U1 intron
- pXF8.198 (13.3 kb)
- Synthetic 5R BDD
- \(h\)FVIII cDNA
- amp
- neo
- \(h\)GH 3' UTS
- TAG (6421)
FIG. 8

pXVEGF.1
(7463 bp)
FIG. 9

Plasma hFVIII (mU/ml)

- ○ SC HSHCM (5 x 1e6 hFVIII cells)
- ◦ SC HSHCM (1 x 1e6 VEGF cells + 5 x 1e6 hFVIII cells)
- ▲ SC HSHCM (2.5 x 1e6 VEGF cells + 5 x 1e6 hFVIII cells)
- ■ SC HSHCM (5 x 1e6 VEGF cells)

Days Post-Implantation
FIG. 11
FIG. 14

- ○ Cells + Microcarriers
- □ Cells + Microcarriers + Heparin-Sepharose Beads
- ▲ Cells + Microcarriers + Heparin-Sepharose Beads w/500ng bFGF
- ■ Cells + Microcarriers + Heparin-Sepharose Beads w/100ng VEGF

Plasma hFVIII (mU/ml) vs. Days Post-Implantation