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(71) Applicant *(for all designated States except US)*: ORGANOGENESIS INC. [US/US]; 15 Dan Road, Canton, MA 02021 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MAYS, Peter, K. [US/US]; Apartment A7, 65 Miller Street, Quincy, MA 02169 (US). KEMP, Paul, D. [US/US]; 7 Meadow Croft Road, Winchester, MA 01890 (US).

(74) Agents: BAKER, Hollie, L. et al.; Hale and Dorr, 1455 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).

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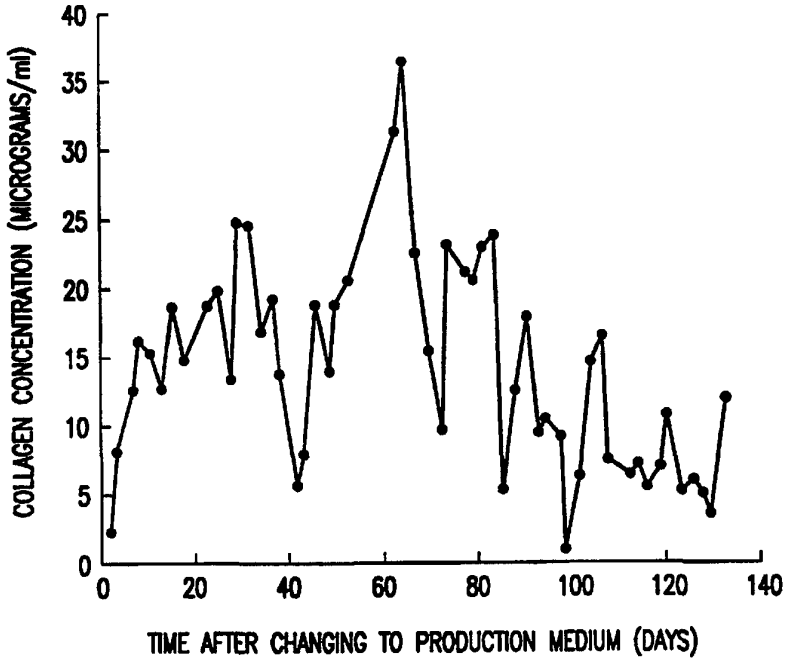

(57) Abstract

This invention is directed to a method for producing collagens from a collagen-producing cell in a cell culturing system. This invention is also directed to the collagens synthesized *in vitro* from the cell cultures. Collagen producing cells are cultured in the presence of an agent to inhibit or interfere with collagen crosslinking. The synthesized collagens are removed from the culture with a solution that maintains the viability of the cells in culture so that collagen synthesis and removal is repeated. The figure illustrates the repeated removal. The collagens produced by this method are useful for biomedical, biotechnology and cosmetic applications.

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Title of the Invention:

COLLAGEN FROM CELL CULTURES

Field of the Invention:

This invention is directed to a method for producing collagen from a collagen-producing cell in a cell culturing system. This invention is also directed to the collagens synthesized *in vitro* from the cell cultures. The synthesized collagens are useful for biomedical, biotechnology, and cosmetic applications.

BACKGROUND OF THE INVENTION

Collagens are the major proteinaceous component of the extracellular matrix of mammalian species. The primary role of collagen is to provide a scaffold to support tissues, Eyre, *Science*, 207:1315-1322 (1980), although a number of other functions have been elucidated for the collagens including roles in cell attachment, cell migration, filtration and morphogenesis. Mays *et al.*, *Biochemical Journal*, 276:307-313 (1991). Collagens are a super-family of closely related proteins sharing some common structural and functional properties, including triple-helical regions which have a repeating triplet of amino acids glycine-X-Y, where X is frequently proline and Y is often hydroxyproline. Hydroxyproline constitutes approximately 12% (w/w) of interstitial fibrillar collagens and is found in only a few other proteins including the complement component C1q, elastin, acetylcholinesterase, conglutinin, type I and type II macrophage scavenger receptors, mannose-binding protein, pulmonary surfactant apolipoproteins A and D, where its prevalence is much lower than in collagens. Mays and Laurent, in *"Molecular Biology of Lung Disease"* (Barnes and Stockley eds.) Blackwell Scientific Publishers, UK; 1994, pages 216-260. Therefore, hydroxyproline is frequently used as an amino acid to identify and quantify collagens. Udenfriend, *Science*, 152:1335-1340 (1966).

Currently, there are nineteen characterized collagens (designated collagens I to XIX) and two less well defined collagens,

which arise from different genetic loci. Kivirikko, *Annals of Medicine*, 25:113-126 (1993); Mays and Laurent, in *"Molecular Biology of Lung Disease", supra.* Myers *et al., Journal of Biological Chemistry* 269:18549-18557 (1994) The different collagens have markedly differing distributions within the body, which are given in table 1. Collagen I is the most prevalent form of collagen in mammalian species and is ubiquitously distributed throughout the body in skin, bone, muscle, tendon, and lung. Collagen IV is found in basement membranes. Collagens VII and XVII collagens are localized to the dermal-epidermal junction. The different collagens are often synthesized by specific cell types, both *in vivo* and *in vitro*, as shown in table 1.

Collagen I, which is considered to be the prototype interstitial fibrillar collagen, is synthesized by a large number of different cell types including fibroblasts, smooth muscle cells, endothelial and epithelial cells. Collagen I is comprised of two different polypeptide alpha-chains, alpha1(I) and alpha2(I), with two alpha1(I)-chains and one alpha2(I)-chain folding together to form a triple-helical collagen molecule. The collagen molecule is synthesized as a larger precursor termed procollagen, where the triple-helical collagen molecule has two large globular extensions which are termed propeptides. In humans, the proalpha1(I)- and proalpha2(I)-chains are encoded by two different genetic loci, proalpha1(I) by the COL1A1 locus on chromosome 17 at q21.3-q22.05 and proalpha2(I) by the COL2A1 locus on chromosome 7 at q21.3-q22.1. The genes for the individual collagen alpha-chains are transcribed to RNA in the nucleus and then spliced to mRNA. The mRNA is transported to the cytoplasm where it is translated and the protein synthesized. The growing collagen polypeptide is translocated to the endoplasmic reticulum by a signal peptide which is proteolytically cleaved coincident with entry to the compartment. In the endoplasmic reticulum, the alpha-chains are post-translationally modified by the enzymes prolyl 4-hydroxylase (EC 1.14.11.2) and lysyl hydroxylase (EC 1.14.11.4), which hydroxylate proline and lysine, respectively, and several sugar residues are placed on the molecule. Kivirikko and Myllya, in *"Extracellular*

Table 1: Tissue Distribution and Cells Producing Collagens

The table gives the principal tissue distribution in mammalian species and examples of some cell types which have been demonstrated *in vitro* to synthesize the collagen.

Collagen	Tissue distribution	Cells capable of synthesizing the collagen *in vitro*	Reference
I	Interstitial	Fibroblast (dermal, human)	Chan et al, Biochem J 1990, 269, 175-181
		Epithelial (type II pneumocyte, rat)	Leheup et al, Lab Invest 1989, 60, 791-807
		Epithelial (kidney, rat)	Creely et al, Am J Pathol 1992, 140, 45-55
		Endothelial (vascular, bovine)	Tseng et al, Eur J Biochem 1982, 122, 355-360
		Ito (rat)	Knittel et al, Gastroenterology 1992, 102, 1724-1735
		Smooth muscle (aortic, rabbit)	Ang et al, Biochem J 1990, 265, 461-469
		Smooth muscle (vascular, human)	Amento et al, Arterio Thromb 1991, 11, 1223-1230
		Smooth muscle (intestinal human)	Graham et al, J Cell Physiol 1995, 162, 225-233
		Chorionic villi (human)	Chamson et al, Prenatal Diagnosis 1995, 15, 165-170
		Hepatocytes (rat)	Guzelian et al, Collagen Relat Res 1981, 1, 83-94
	Bone	Osteoblasts (murine)	Quarles et al, J Bone Miner Res 1992, 7, 683-692
II	Cartilage	Chondrocytes (vertebral , chick)	Pacifici and Iozzo, J Biol Chem 1988, 263, 2483-2492
		Chondrocytes (costal, human)	Sandell et al, J Cell Biol 1991, 114, 1307-1319
		Periosteal (bovine)	Izumi et al, J Bone Miner Res 1992, 7, 115-121
III	Interstitial	Fibroblast (dermal, human)	Chan et al, Biochem J 1990, 269, 175-181
		Epithelial (type II pneumocyte, rat)	Leheup et al, Lab Invest 1989, 60, 791-807
		Epithelial (kidney, rat)	Creely et al, Am J Pathol 1992, 140, 45-55
		Endothelial (vascular, bovine)	Tseng et al, Eur J Biochem 1982, 122, 355-360
		Ito (rat)	Knittel et al, Gastroenterology 1992, 102, 1724-1735
		Smooth muscle (aortic, rabbit)	Ang et al, Biochem J 1990, 265, 461-469
		Smooth muscle (intestinal human)	Graham et al, J Cell Physiol 1995, 162, 225-233
		Chorionic villi (human)	Chamson et al, Prenatal Diagnosis 1995, 15, 165-170

Table 1 (continued)

IV	Basement membrane	Epithelial (type II pneumocyte, rat)L	Leheup et al, Lab Invest 1989, 60, 791-807
		Epithelial (kidney, rat)	Creely et al, Am J Pathol 1992, 140, 45-55
		Myoepithelial (mammary, rat)	Warburton et al, J Cell Physiol 1986, 128, 76-84
		Endothelial (vascular, bovine)	Tseng et al, Eur J Biochem 1982, 122, 355-360
		Smooth muscle (aortic, rat)	Hospelhorn et al, Matrix 1992, 12, 352-361
		Smooth muscle (vascular, human)	Tan et al, Matrix 1991, 11, 380-387
		Ito (rat)	Knittel et al, Gastroenterology 1992, 102, 1724-1735
		Hepatocytes (rat)	Loreal et al, Am J Pathol 1993, 143, 538-544
		Chorionic villi (human)	Chamson et al, Prenatal Diagnosis 1995, 15, 165-170
V	Interstital	Fibroblast (dermal, human)	Chan et al, Biochem J 1990, 269, 175-181
		Epithelial (type II pneumocyte, rat)	Leheup et al, Lab Invest 1989, 60, 791-807
		Epithelial (kidney, rat)	Creely et al, Am J Pathol 1992, 140, 45-55
		Endothelial (vascular, bovine)	Tseng et al, Eur J Biochem 1982, 122, 355-360
		Smooth muscle (aortic, rabbit)	Ang et al, Biochem J 1990, 265, 461-469
VI	Interstitial	Fibroblast (dermal, bovine)	Kielty et al, Biochem J 1990, 272, 787-795
		Fibroblast (nuchal ligament, bovine)	Kielty et al, Biochem J 1990, 272, 787-795
		Epithelial	
		Endothelial (vascular human)	Tan et al, Matrix 1991, 11, 380-387
		Smooth muscle (vascular human)	Tan et al, Matrix 1991, 11, 380-387
VII	Epidermal-dermal junction	Fibroblasts (dermal)	Konig and Bruckner-Tuderman, J Cell Biology 1992, 117, 679-685
		Epithelial (keratinocytes)	Ryynanen et al, J Clin Invest 1992, 89, 163-168
VIII	Descemet's membrane Tunica intima of blood vessels, periosteum, perichondrium	Vascular endothelial	Sage et al, Lab Invest, 1984, 50, 219-231
		Corneal endothelial	Benya and Padilla J Biol Chem 1986, 261, 4160-4169
		Human mast cells	Ruger et al, Int J Exp Path 1994, 75, 397-404
IX	Cartilage	Chondrocytes (vertebral, chick)	Pacifici, Biochem J 1990, 272, 193-199

Table 1 (continued)

X	Cartilage	Chondrocytes	Bonen and Schmid J Cell Biol 1991, 115, 1171-1178
XI	Cartilage	Chondrocytes (Chick sterna)	Thom and Morris, J Biol Chem 1991, 266, 7262-7269
XII	Interstitial	Fibroblasts (Chick embryo skin) Epithelial (WISH - from human amniotic epithelium)	Koch et al, Eur J Biochem 1992, 207, 847-856. Lunstrum et al, J Biol Chem 1992, 267, 20087-20092
XIII 16922-16928	Interstitial	Endothelial (human)	Pihlajaniemi and Tamminen, J Biol Chem 1990, 265,
	Placenta	Fibrosarcoma (HT-1080, human)	Pihlajaniemi and Tamminen, J Biol Chem 1990, 265,
16922-16928		Fibroblasts (human skin)	Juvonen et al, J Biol Chem 1992, 267, 24700-24707
XIV	Interstitial	Fibroblasts	
XV	Basement membrane		
XVI	Interstitial	Fibroblast (Dermal) Epithelial (Keratinocyte)	Pan et al, Proc Natl Acad USA 1992, 89, 6565-6569 Pan et al, Proc Natl Acad USA 1992, 89, 6565-6569
XVII	Dermal-epidermal junction	Fibroblasts (NIH-3T3 mouse transormed) Epithelial (Mouse Balb/K keratinocytes)	Li et al, J Biol Chem 1993, 268, 8825-8834 Li et al, J Biol Chem 1993, 268, 8825-8834
XVIII	Basement membrane		
XIX	Interstitial	Rhabdomyosarcoma (human)	Inoguchi et al, J Biochem 1995, 117, 137-146

Matrix Biochemistry" (Piez & Reddi eds.) Elsevier, 1984, pages 83-118. The C-termini of two alpha1(I)- and one alpha2(I)-chains are brought together, probably by a molecular chaperone, colligin, which is a member of the heat shock family of proteins. Clarke *et al., Journal of Cell Biology,* 121:193-199 (1993); Nakai *et al,. Journal of Cell Biology,* 117:903-914 (1992). This initial registration of the C-termini is stabilized by disulfide cross-linking and the alpha-chains fold into a triple-helix from the C- to the N-terminus. Gelman *et al., Journal of Biological Chemistry,* 254:11741-11745 (1979). This folding may only occur once the Y-position prolines are fully hydroxylated to hydroxyproline by the action of prolyl 4-hydroxylase. Once folded, the procollagen molecule is transported to the Golgi apparatus where it is packaged into a secretory vesicle. The procollagen is secreted into invaginated folds at the cell surface. At the time of secretion, or just after, the procollagen peptides are proteolytically cleaved by specific C- and N-proteinases. Hojima *et al., Journal of Biological Chemistry,* 260:15996-16003 (1985); Hojima *et al., Journal of Biological Chemistry,* 264:11336-11345 (1989). The cleaved propeptides are believed to negatively-feedback to inhibit procollagen gene expression. Wu *et al., Journal of Biological Chemistry,* 266:2983-2987 (1991); Fouser *et al., Proceedings of the National Academy of the Sciences of the United States of America,* 88:10158-10162 (1991). The triple-helical collagen molecule liberated by propeptide cleavage contains two short non-helical extensions at either end of the molecule, termed telopeptides. The collagen molecules spontaneously interact to form a quarter-stagger arrangement at the invaginated cell surface. The telopeptides play a critical role in stabilizing the quarter-staggered arrangement of the extracellular collagens. Once in a quarter-staggered arrangement, the enzyme lysyl oxidase (protein lysine 6-oxidase, EC 1.4.3.13) oxidizes the epsilon amino groups of a specific lysine or hydroxylysine residue in both the C- and N-telopeptides to an aldehyde carbonyl, alpha-aminoadipic-delta-semialdehyde. The aldehyde carbonyl is highly reactive and it spontaneously undergoes condensation with adjacent amino groups of unreacted lysine residues to form a covalent

crosslink. If the lysine residue is in a neighboring collagen molecule, then the resulting crosslink will stabilize the quarter-staggered collagen fibril. Over time these crosslinks are further stabilized by the Amadori rearrangement and the collagen becomes progressively less soluble. Reiser *et al., FASEB Journal* 6:2439-2449 (1992).

The collagen fibrils formed at the cell surface are deposited into the extracellular matrix where they interact further with other collagen fibrils and other components of the extracellular matrix to form the functional extracellular matrix. The collagen fibrils often consist of more than one collagen type, and the composition of the fibrils is important for determining fibrillogenesis and tissue function. Lapiere *et al., Connective Tissue Research* 5:21-29 (1977), Andrikopoulos *et al., Nature Genetics* 9:31-36 (1995). Further, extracellular matrix packing may be regulated by other non-collagenous components of the extracellular matrix (e.g. dermatan sulfate). Hahn and Birk, *Development* 115: 383-393 (1992). Collagen fibers, in tissues rich in collagen I (e.g. tendon), are characterized by their 67nm banding pattern when visualized by heavy metal staining under the electron microscope.

During fibrillogenesis and crosslinking the nascent collagen fibrils and fibers are able to be extracted by different chemical solutions. Prior to the formation of covalent crosslinks the collagens are extractable with dilute salt solutions. After the formation of covalent crosslinks and prior to the Amadori rearrangement the collagen is extractable in dilute acetic acid. Once the Amadori rearrangement occurs the collagen becomes insoluble, and can only be solubilized by enzymatic action (e.g. pepsin, matrix metalloproteinases) which degrade the collagen. These observations were first made in tissues of animals; where there were pools of collagens with differing solubilities, and upon radiolabeling the more soluble pools contained greater amounts of radiolabeled material than less soluble pools, suggesting that they were more recently synthesized. Nimni *et al,. Biochemical Journal* 102:143-147 (1967). Subsequently, these observations have been

extended to cells in culture. Chan *et al., Biochemical Journal* 269:175-181 (1990).

Presently there are nineteen characterized collagens, each with specific properties, structure and functions. Table 1. van der Rest and Garrone, *FASEB Journal* 5:2814-2823 (1991), Kielty *et al* in *"Connective Tissue and Its Heritable Disorders: Molecular, Genetic and Medical Aspects"* (Royce and Steinmann,eds.). Wiley-Liss, 1993, pages 103-147. Collagens II, III, V and XI share a similar structure and crosslinking pattern to collagen I, the prototype interstitial fibrillar collagen, as described above. Collagen IV is found in basement membranes and has a large globular C-terminal domain, a long collagenous domain of 350nm and a small N-terminal domain in the mature protein, which packs into a filamentous network, which has been described as a 'chicken-wire' pattern. Collagen IV crosslinking is by both reducible (disulfide) bonds and non-reducible (lysine-derived, lysyl oxidase-mediated) bonds. Collagen VI which forms a microfibrillar network is disulfide crosslinked both intra- and inter-molecularly. Collagen VII, localized to anchoring fibrils at the dermal-epidermal junction, is a large molecule with both globular and interrupted collagen triple-helical regions, it is believed to associate via disulfide bonds in a tail-to-tail assembly. Less is known about crosslinking and molecular packing in the other collagens, but it is clear that the various collagens crosslink by different mechanisms dependent on their structure and function. Also there is intermolecular crosslinking between different collagens in a collagen fibril, e.g. between collagen II, a fibrillar collagen, and collagen IX, a fibril-associated collagen with interrupted triple helices (FACIT), which are lysine-derived lysyl oxidase-mediated crosslinks. Eyre *et al., FEBS Letters* 220:337-341 (1987).

The enzyme lysyl oxidase is synthesized intracellularly as a 411 amino acid precursor. The gene encoding lysyl oxidase has been fully characterized and has seven exons in about 19kb of DNA localized at 5q23.3-31.2. The transcribed gene product gives rise to some variably-sized mRNA species, due to alternate polyadenylation site usage, of about 2.0-5.5kb which are abundantly expressed in

connective tissue-rich cells both *in vivo* and *in vitro*. The translated gene product is a 46.6kDa proenzyme, which is proteolytically activated by metalloproteases to give an active enzyme with a molecular weight of about 32kDa. The enzyme has been expressed in a cell-free *in vitro* expression system. Trackman *et al., Journal of Biological Chemistry* 267:8666-8671 (1992). The activated enzyme oxidizes lysine and hydroxylysine residues in collagen and elastin. In collagen I only specific lysine and hydroxylysine residues in the telopeptides are modified, whereas in elastin about 30 of the 48 lysine residues per 1000 amino acids are oxidized. In collagen I lysyl oxidase is only capable of oxidatively deaminating the lysine and hydroxylysine residues in the telopeptides, once the molecule has achieve a quarter-staggered arrangement. The enyzme is believed to associate with homologous sequences near the C- and N-terminal ends of the triple-helices of collagen molecules which are proximal to the lysine or hydroxylysine residues in the telopeptides of adjacent quarter-staggered molecules. Siegel, *International Review of Connective Tissue Research* 8:73-118 (1979). The enzyme requires at least two cofactors, copper and the quinone of peptidyl trihydroxyphenylalanine (TOPA). A number of agents have been identified which are capable of inhibiting the enzyme, examples of such compounds are: beta-aminopropionitrile, beta-bromoethylamine, beta-nitroethylamine, benzylamines, diamine analogs, isoniazid, iproniazid, *cis*-diaminocyclohexane, hydrazines, semicarbizides and dithiothreitol (US Patent # 4,997,854). Collagen synthesized in the presence of a lysyl oxidase inhibitor is soluble in cold dilute neutral salt solutions. This soluble collagen if allowed to form fibrils, may be acted upon by lysyl oxidase to crosslink the fibrils, and this approach has been used as an assay for lysyl oxidase. Prockop and Tuderman, *Methods in Enzymology*, 82:305-319 (1982).

Other agents are known to interfere with the formation and/or maturation of lysine-derived lysyl oxidase-mediated crosslinks in collagens, including D-penicillamine and heparin. D-penicillamine inhibits crosslink formation by blocking the aldehyde carbonyl groups which are formed following oxidative deamination of the

lysine/hydroxylysine residues in the telopeptides. If this collagen is subsequently extracted and purified, to remove the D-penicillamine, stable insoluble collagen fibrils will form *in vitro*, indicating that the aldehyde carbonyl groups have been exposed and are available to form covalent crosslinks. Nimni *et al.*, in *"Chemistry and Molecular Biology of the Intercellular Matrix"* (Balazs ed) Academic Press, New York, NY, 1970, pages 417-430. Heparin inhibits collagen crosslinking by interfering with the ability of the collagen molecules to form fibrils, thus the collagen molecules are not in the quarter stagger arrangement needed for lysyl oxidase activity, and also heparin reduces the binding of lysyl oxidase to collagen I by about forty percent. Gavriel and Kagan, *Biochemistry* 27:2811-2815 (1988)

There is a need in medicine for biomaterials that are versatile and compatible with human tissues. Collagen has several unique properties which make it a good candidate for medical implant applications, the most apparent of which is that collagen is a natural biomaterial. It has been shown that collagen based implants may be remodeled by the host after implantation. Kato *et al.*, *Bone and Joint Surgery,* 73:561-574 (1991).

Collagen has been used in a large number of biomedical, biotechnical and cosmetic uses. (Stenzel *et al., Annual Review of Biophysics and Bioengineering,* 3:231-253 (1974). Collagen medical devices have been designed for use in cardiovascular surgery, plastic surgery, ophthalmology, orthopedics, urology, thoracic surgery, abdominal surgery, otology, and neurosurgery, as well as being designed as a drug delivery system, hemostatic agent, anti-adhesive and adhesive. Chvapil M., Kronenthal R.L. and van Winkle W., *International Review of Connective Tissue Research,* 6:1-61 (1973). Examples of the commercial uses of collagen include insoluble hemostatic sponges (COLLASTAT, Integra Life Sciences, NJ) or flour (U.S. 3,443,261), extruded collagen sutures (U.S. 3,114,593), collagenous tissue equivalents (U.S. 4,485,096), fibrillar constructs (U.S. 5,256,418), as solubilized, injectable, atelopeptide collagen for tissue augmentation (U.S. 3,949,073), as a collagen eye-shield to delivery antibiotics to the eye (Phinney *et al., Archives of*

Ophthalmology 106:1599-1604 (1988), as an implantable collagen-poly(HEMA) hydrogel to delivery drugs (Jeyanthi *et al., Journal of Pharmacy and Pharmacology* 43:60-62 (1991) and as collagen microparticles to delivery drugs (Rossler *et al., Journal of Microencapsulation* 12:49-57 (1995). The vast majority of collagens used in the medical device, pharmaceutical and cosmetic applications today are obtained from animal sources. Collagens may be extracted from collagenous rich tissues from a number of different phyla, including cattle, pigs, rats, poultry, and fish. The extraction process may be either enzymatic, where pepsin is frequently employed to cleave the crosslinked telopeptides from the collagenous triple-helical region of the molecule, thereby liberating atelopeptide collagenous triple-helices, or by extraction of the collagen in a weak acid. These extracted animal collagens have the disadvantage that they are not derived from human sources, and could also be contaminated with non-collagenous material.

To overcome the problem of cross-species of collagens several approaches have been taken to obtain commercially viable sources of human collagen. One method is to extract collagen from human placenta as described in U.S. 5,002,071 (Harrell, C.; Research Development Foundation). The supply of human placentae are variable, however, and there is the potential for the transmission of communicable diseases and the broader ethical issues which limit the overall commercialization of this approach.

In another method, collagens derived from recombinant cells in culture are being developed to generate human collagens from genetically-engineered genes PCT application WO 93/07889. For example, the gene for procollagen II has been inserted into HT-1080 cells and expressed as a normal procollagen II molecule into the cell medium. Fertala *et al., Biochemical Journal,* 298:31-37 (1994). The HT-1080 cells express only collagen IV and other basement membrane components, thus these cells are capable of synthesizing a collagen (i.e. they express all post-translational enzymes), but they do not constitutively express interstitial collagens such as collagens I and II, and so are a useful mammalian cell line to produce recombinant procollagens. Similarly, these workers have produced a

homotrimeric procollagen I, containing only proalpha1(I)-chains in the same cell line. Geddis and Prockop, *Matrix,* 13:399-405 (1993). The limitation of this process is that the procollagen must be processed by proteolytic cleavage of the two propeptides to collagen and then purified from other medium components, to liberate a collagen with intact telopeptides. Alternatively the procollagens in the cell culture medium may be treated with pepsin to proteolytically remove the propeptides and the telopeptides. Others have used similar approaches using different cell types for other collagens, for instance Greenspan and coworkers have expressed the alpha2-chain of human collagen V [α2(V)] in Chinese hamster lung (CHL) cells which do not produce this collagen alpha-chain, so as to produce a chimeric heterotrimeric collagen V molecule comprising one human α2(V)-chain and two hamster α1(V)-chains in triple-helical form. Greenspan *et al., Journal of Biological Chemistry* 264:20683-20687, 1989. A truncated form of chick collagen XII, comprising the C-terminal portion including the NC1 and COL1 domains and part of the NC2 domain, has been expressed using a recombinant minigene for the alpha1-chain of chicken collagen XII [α1(XII)] and expressing it in HeLa cells which produce no collagen I or XII. The expression of this construct resulted in the formation of a triple-helical truncated form of human collagen XII in the cell culture medium used to grow these cells. Mazzorana *et al., Journal of Biological Chemistry* 268: 3029-3032, 1993. Another genetic engineering approach has been to insert a baculovirus vector containing the necessary genes for procollagen synthesis into Sf9 insect cells to produce recombinant human collagen. Vuori *et al., Proceedings of the National Academy of Sciences of the United States of America*, 89:7467-7470 (1992). The limitation of this system is that not only do the procollagen genes have to be inserted, but also all the necessary post-translational genes must be expressed, such as prolyl 4-hydroxylase, to allow folding and secretion to occur. This approach also requires purification of the procollagen from the cell culture medium and then subsequently processing of the procollagen to collagen.

In yet another approach, transgenic animals are being developed to produce procollagen. It is has been demonstrated that genes for human therapeutic proteins may be cloned, genetically engineered and inserted into a transgenic animal in such a way that the transgenic animal expresses the protein in mammary epithelial cells and secretes the recombinant human protein into the lactated milk, thereby enabling the protein to be recovered from the animals milk (U.S. 4,873,316; Lonberg, *et al.,* Biogen Inc.). More recently a method has been proposed for generating recombinant human collagens by utilizing such an approach (PCT WO 94/16750; Berg, Collagen Corporation). Although transgenic animals may be able to produce human procollagen, further purification and processing of the procollagen would still be required to purify it from the milk proteins and to remove the propeptides.

It was previously known that cells grown in culture supplemented with ascorbate would produce collagens. Goldberg *et al., Experimental Cell Research*, 31:444-447 (1963); Schwartz *et al., Journal of Cell Biology,* 92:462-470 (1982); Grinnell *et al., Experimental Cell Research,* 181:483-491 (1989). However, most of the synthesized collagen is secreted as procollagen into the cell culture medium and is not processed to collagen. Limeback and Sodek, *European Journal of Biochemistry,* 100:541-550 (1979). A smaller fraction of the synthesized procollagen is processed into collagen and deposited into the pericellular matrix around the cells. The deposited collagens are crosslinked by the action of the enzyme, lysyl oxidase, and form an insoluble collagen matrix.

Collagen synthesis in culture is dependent on both the growth state of the cells and on the length of time in culture. During periods of rapid growth, the cells synthesize very little collagen, but once confluent, the cells markedly increase their collagen synthesis. Booth *et al., Biochemica Biophysica Acta,* 607:145-160 (1980); Steinberg, *Laboratory Investigation*, 39:491-496 (1978). After a period of one to three weeks, collagen synthesis and deposition into the pericellular matrix decreases markedly to negligible levels. Chan *et al., Biochemical Journal*, 269:175-181 (1990); Grinnell *et al., Experimental Cell Research*, 181:483-491

(1989). Also secretion of procollagen into the cell culture medium falls dramatically. Previous studies of collagen synthesis in culture have used lysyl oxidase inhibitors to inhibit collagen crosslinking, thereby allowing some of the collagen deposited in the extracellular matrix to be dissolved in a cold neutral saline solution over varying periods of time from 16 hours to 48 hours. After the cold neutral saline solution extraction, the remainder of the cell layer was scraped into acetic acid (0.5M), Lamande and Bateman, *Matrix,* 13:323-330 (1993) or sodium hydroxide (0.2M), Bankowski & Dabrowski, *Acta Biochemica Polonica*, 27:405-411 (1980), to solubilize some of the collagens which were partially crosslinked. The application of fibroblasts grown in culture to produce human collagen in commercially usable quantities has not been possible given the problems with maintaining a continual high level of collagen synthesis, and having methods to harvest and process the procollagen to collagen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph showing different collagens separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, as described in Example 3. Lane assignments are: Lane 1 (on the left), high molecular weight standards (Bio-Rad Laboratories, Hercules, CA); lane 2, pepsin treated human collagen extracted from the cell layer of Example 1 with Dulbecco's phosphate-buffered saline; lane 3, human collagen extracted from the cell layer of Example 1 with Dulbecco's phosphate-buffered saline; lane 4, bovine collagen I treated with pepsin (Organogenesis Inc., Canton, MA); lane 5, bovine collagen I (Organogenesis Inc., Canton, MA); lane 6, pepsin treated human collagen I (Sigma Chemical Co, St Louis, MO) retreated with pepsin; lane 7 pepsin treated human collagen I (Sigma Chemical Co, St Louis, MO); lanes 8 and 9, high molecular weight standards (Bio-Rad Laboratories, Hercules, CA); lane 10, pepsin (100µg/ml) in aqueous acetic acid (0.5M).

Figure 2 shows the concentration of the collagen extracted from the cell layer with Dulbecco's phosphate-buffered saline, as described in Example 1. The collagen concentration was determined

by measuring the amount of hydroxyproline in the Dulbecco's phosphate-buffered saline as described in Example 4. The first cold cycle was two days after the medium was changed to production medium and the last cold cycle shown in figure was 133 days after the medium was changed to production medium.

Figure 3 is a photograph showing the concentrated human collagen separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described in Example 8. Lane assignments are: Lane 1 (on left) high range molecular weight standard (Bio-Rad Laboratories, Hercules, CA). From top: myosin, 200kDa; *B*-galactosidase, 116.5kDa; phosphorylase B, 97.4kDa; serum albumin, 66.2kDa.; lane 2, concentrated human collagen from example 7, 21µg; lane 3, concentrated human collagen from Example 8, 30µl loading; lane 4, concentrated human collagen from Example 8, 15µl loading; lane 5, concentrated human collagen from Example 8, 10µl loading; lane 6, concentrated human collagen from Example 8, 8µl loading; lane 7, concentrated human collagen from Example 8, 6µl loading; lane 8, concentrated human collagen from Example 8, 4µl loading; lane 9, concentrated human collagen from Example 7, 14µg loading; lane 10, concentrated human collagen from Example 7, 5.6µg loading.

Figure 4 is a photograph of an electron micrograph, at 55,000x magnification, of a DFC construct prepared as described in Example 9. The photograph shows the regular D-period banding of the collagen fibrils.

Figure 5: Photograph of a human collagen sponge, prepared as described in Example 10, showing surface texture at 8X magnification (sponge dimensions: 4mmX30mm).

Figure 6 is a photograph showing cyanogen bromide-derived peptides from concentrated human collagen, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, demonstrating the presence of collagen III, as described in Example 12. Lane assignments: Lane 1, blank (loading buffer added); lane 2, broad molecular weight marker (Bio-Rad Laboratories, Hercules, CA); lane 3, concentrated human collagen from Example 1 undigested (12.92µg); lane 4, concentrated human collagen from Example 1 digested with cyanogen bromide (32.7µg); lane 5, human collagen I

digested with cyanogen bromide (29.4µg, Sigma type VIII, pepsin extracted from human placenta, Sigma Chemical Company, St. Louis, MO); lane 6, concentrated human collagen extracted from the cell layer digested with cyanogen bromide (32.7µg); lane 7, human collagen III, digested with cyanogen bromide (29.4µg, Sigma type X, pepsin extracted from human placenta, Sigma Chemical Company, St. Louis, MO).
KEY: A, alpha1(I)CB8;B, alpha1(III)CB5; C, alpha1(I)CB6;
D, alpha1(I)CB6'

Figure 7 is a photograph showing cyanogen bromide-derived peptides from concentrated human collagen, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, demonstrating the presence of telopeptides, as described in Example 13. Lane assignments: Lanes 1 and 2: blank (loading buffer added); lane 3: broad molecular weight marker (Bio-Rad Laboratories, Hercules, CA); lane 4, human collagen I, 8ug, digested with cyanogen bromide (Sigma type VIII, pepsin extracted from human placenta, Sigma Chemical Company, St. Louis, MO); Lane 5, concentrated human collagen from the cell layer of example 8, digested with cyanogen bromide, lane 6, human collagen concentrated from the cell layer of example 8, digested with pepsin prior to digestion with cyanogen bromide digestion; Lane 7, human collagen I digested with cyanogen bromide (8µg, Sigma type VIII, pepsin extracted from human placenta, Sigma Chemical Company, St. Louis, MO); lane 8, human collagen III digested with cyanogen bromide (10µg, pepsin extracted from human placental villi, Southern Biotechnology Associates, Birmingham, AL); lanes 9 and 10: blank (loading buffer added). KEY: A, alpha1(I)CB6; B, alpha1(I)CB6'

Figure 8 is a photograph showing the presence of collagen alpha-chains separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the Dulbecco's phosphate buffered saline harvests and the acetic acid extract of the cell layer of cell strain HDF B116, as described in example 14. Lane assignments are: Lane 1 (on the left), blank (loading buffer added); lane 2, high range molecular weight standard (Bio-Rad Laboratories Hercules, CA). From top: myosin, 200kDa; *B*-galactosidase, 116.5kDa; phosphorylase

B, 97.4kDa; serum albumin, 66.2kDa; lane 3, Dulbecco's phosphate buffered saline extract of the HDF B116 cell layer at 7 days in production medium; lane 4, Dulbecco's phosphate buffered saline extract of the HDF B116 cell layer after 21 days in production medium; lane 5, Dulbecco's phosphate buffered saline extract of the HDF B116 cell layer after 28 days in production medium; lane 6, Dulbecco's phosphate buffered saline extract of the HDF B116 cell layer after 35 days in production medium; lane 7, Dulbecco's phosphate buffered saline extract of the HDF B116 cell layer after 42 days in production medium; lane 8, acetic acid soluble collagen cell layer from HDF B116 cell layer; lane 9, human collagen I (12μg, Sigma type VIII, pepsin extracted collagen from human placenta. Sigma Chemical Company, St Louis, MO); lane 10, blank (loading buffer added).

Figure 9 is a photograph showing the presence of collagen alpha-chains separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the Dulbecco's phosphate buffered saline harvests and the acetic acid extract of the cell layer of cell strain HDF B117, as described in example 15. Lane assignments are: Lane 1 (on the left), blank (loading buffer added); lane 2, high range molecular weight standard (Bio-Rad Laboratories Hercules, CA). From top: myosin, 200kDa; *B*-galactosidase, 116.5kDa; phosphorylase B, 97.4kDa; serum albumin, 66.2kDa; lane 3, Dulbecco's phosphate buffered saline extract of the HDF B117 cell layer after 7 days in production medium; lane 4, Dulbecco's phosphate buffered saline extract of the HDF B117 cell layer after 14 days in production medium; lane 5, Dulbecco's phosphate buffered saline extract of the HDF B117 cell layer after 21 days in production medium; lane 6, Dulbecco's phosphate buffered saline extract of the HDF B117 cell layer from after 28 days in production medium; lane 7, Dulbecco's phosphate buffered saline extract of the HDF B117 cell layer after 35 days in production medium; lane 8, acetic acid soluble collagen cell layer from HDF B117 cell layer; lanes 9 and 10, blank (loading buffer added).

Figure 10 is a photograph showing the presence of collagen alpha-chains separated by sodium dodecyl sulfate polyacrylamide

gel electrophoresis of the Dulbecco's phosphate buffered saline harvests and the acetic acid extract of the cell layer of cell strain SAF 012A, as described in example 16. Lane assignments are: Lane 1 (on the left), blank (loading buffer added); lane 2, high range molecular weight standard (Bio-Rad Laboratories Hercules, CA). From top: myosin, 200kDa; *B*-galactosidase, 116.5kDa; phosphorylase B, 97.4kDa; serum albumin, 66.2kDa; lane 3, Dulbecco's phosphate buffered saline extract of the SAF 012A cell layer after 21 days in production medium; lane 4, Dulbecco's phosphate buffered saline extract of the SAF 012A cell layer after 28 days in production medium; lane 5, Dulbecco's phosphate buffered saline extract of the SAF 012A cell layer after 35 days in production medium; lane 6, acetic acid soluble collagen cell layer from SAF 012A cell layer; lanes 7-10, blank (loading buffer added).

SUMMARY OF THE INVENTION

By this invention, the ability to produce collagen from a cell culturing system in heretofore unavailable quantities and simplicity of processing is demonstrated. Particularly, this invention relates to novel collagens, especially human collagens, produced from an *in vitro* cell culturing system. The method of this invention is a repeated cell culturing method utilizing a collagen-producing cell with a culture medium containing an agent to interfere with collagen crosslinking, such as that the thus produced collagens remains non-crosslinked. The produced collagens can be recovered by dissolving them in a salt solution and separating the salt solution containing the dissolved collagens from the collagen-producing cultured cells. The same collagen-producing cells, are then recultured using fresh medium, thus providing for a repeated culturing and collagen production cycling system. By culturing the collagen-producing cells under conditions such that non-crosslinked collagens are synthesized, removing the culture medium, washing the cells with a salt solution to dissolve and recover the collagens, and then reculturing the cells, the same collagen-producing cells will continue to secrete procollagens, process them to collagens and

deposit them within the cell layer which can then be recovered in successive repetitions of this method.

The novel collagens, especially human collagens, are initially produced from the *in vitro* cell culturing system as non-crosslinked, after interfering with collagen crosslinking. By using various recovery techniques, the collagen can be used in biomedical, biotechnology, and cosmetic applications. The extracted collagen can be treated with enzymes, such as lysyl oxidase, to allow it to crosslink. Further, the non-crosslinked collagen may be crosslinked by chemical means such as carbodiimides, glutaraldehyde, sugars and UV irradiation. Further, the recovered collagen can be treated with enzymes such as pepsin or trypsin to form atelopeptide collagen. Further, the procollagen peptides can be separately isolated and recovered from the spent culture medium. Further the procollagen in the cell culture media may be recovered, treated with pepsin to remove the propeptides and telopeptides to produce triple-helical atelopeptide collagen. Further, at termination of the culture, collagen can also be recovered from the cell layer by solubilizing the pericellular collagens in the cell layer with dilute acetic acid. Further, at termination of the culture, collagens can also be recovered from the cell layer by treatment with pepsin to liberate the triple-helical collagen molecules without the crosslinked telopeptides.

DETAILED DESCRIPTION OF THE INVENTION

The *in vitro* cell culturing system of this invention advantageously makes use of the ability of a cell in culture to synthesize collagen as explained with the actual cell synthesis of a collagen I.

The method of this invention involves a cell culturing system in which collagen-producing cells are cultured in a culture medium containing an agent to interfere with collagen crosslinking to inhibit or markedly reduce the formation of intra- and inter-molecular crosslinks in the cell synthesized collagen and retaining the collagen's solubility in cold dilute neutral salt solutions. Once the cells in culture have synthesized the collagen, and secreted it, the

thus produced non-crosslinked collagen can be removed by solubilization in cold dilute neutral salt solutions, while retaining the culture's ability to synthesize collagen. The collagen is recovered by removing the cell culture medium from the cell layer and washing the cell layer with a cold dilute neutral salt solution. As the synthesized collagen is non-crosslinked, it is soluble in the cold dilute neutral salt solution and can then be subsequently recovered from the solution. Fresh cell culture medium with the agent to interfere with collagen crosslinking is then added to the same cell layer and the cycling of the cell culture system, with incubation, culture medium removal, cold dilute neutral salt solution wash is repeated. This cycling of steps may be repeated as long as the collagen-producing cells continue to produce collagen. Given the reports on the down-regulation of collagen production by cells in culture with time (Chan *et al., Biochemical Journal,* 269:175-181 (1990); Grinnell *et al., Experimental Cell Research,* 181:483-491 (1989)) the ability to repeat the cycling of steps in the cell culture system to continue production of collagen was unexpected.

Any cell that is capable of producing or synthesizing collagens may be used in the method of this invention. Fibroblast cells produce predominantly collagen I. Human fibroblast cell strains can be derived from a number of sources, including, but not limited to human neonate male foreskin, human dermal fibroblasts, human Achilles fibroblasts, human pulmonary fibroblasts, human urethra fibroblasts and human intestinal fibroblasts. The human cells need not be limited to fibroblasts, but may include, although not limited to, human smooth muscle cells, human endothelial cells, and human epithelial cells of pulmonary and dermal origin. Furthermore, the cells are not limited to cells from human sources, cells from other mammalian species including, but not limited to: equine, canine, porcine, bovine, ovine sources may be used, as can cells from other phyla, including but not limited to, fish, birds, and invertebrates. In addition, cells which are spontaneously, chemically or virally transformed may also be used in this invention, but it has been shown by several investigators that transformed cells produce less collagen in culture Hajnal *et al., Advances in Enzyme Regulation*

33:267-280 (1993); Kopp, *et al., International Journal of Cancer* 60:275-279 (1995); Kreig *et al., Experimental Cell Research* 125:23-30 (1980); Lefebrve *et al., Journal of Cell Biology* 128:239-245 (1995). These cells may be derived from tissues of human, other mammalian species or other phyla. Further, the cells may be recombinant or genetically-engineered cells that express collagens which are either 'normal' but expressed at high levels or modified in some way to make the collagen therapeutically advantageous. For example, to perform this the nucleotide sequence coding for the desired collagen may be isolated either as genomic DNA, from either cells or tissues, or cDNA, generated from mRNA from cells or tissues expressing the desired collagen. The desired construct can either be driven by the endogenous 5' promoter or by an exogenous promoter, which would be supplied by an expression vector. Modified collagens may be engineered by appropriate site-directed mutagenesis. The desired nucleotide sequence is cloned, by inserting it into an expression vector, introducing the composite DNA molecule into a host cell in which it may replicate, and then subsequently selecting clones with the appropriate target nucleotide sequence. The vector containing the appropriate nucleotide sequence is isolated and linearized prior to cell transfection. The linearized vector is transfected into cells, containing the appropriate post-translational machinery, thereby allowing collagen synthesis to occur, along with a marker gene, such as one conferring resistance to an antibiotic, if not part of the vector construct. The transfected cells are isolated by using the antibiotic to select those cells which have resistance to the antibiotic and therefore those which have been transfected with the marker gene. The transfected cells can either be pooled or clonally developed into cell lines which express the desired nucleotide sequence as a protein. These procedures are generally known in the art, and are described in Sambrook *et al, Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). All of the above-mentioned types of cells are included within the definition of a "collagen-producing cell" as used in this invention.

Similarly within the scope of the invention is a method to harvest collagens produced from cultured cells where pharmacological agents have been added to the culture to alter the nature, amount or type of the collagen secreted. These agents may include polypeptide growth factors, transcription factors or inorganic salts to up-regulate collagen transcription. Examples of polypeptide growth factors include transforming growth factor-beta1 and tissue-plasminogen activator, both of which are known to upregulate collagen synthesis. Raghow *et al., Journal of Clinical Investigation,* 79:1285-1288 (1987); Pardes *et al., Journal of Investigative Dermatology,* 100:549 (1993). An example of an inorganic salt which stimulates collagen production is cerium. Shivakumar *et al., Journal of Molecular and Cellular Cardiology* 24:775-780 (1992).

The major collagen secreted by fibroblast cells is collagen I, however, this invention is not to be limited to only collagen I. For instance, other collagens (e.g. collagens II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX) may be produced by use of appropriate cell types (see Table I, for examples) and/or pharmacological manipulation, and/or genetic engineering. Pharmacological manipulation would be used to elicit the expression of different collagens from a collagen producing cell and/or to prevent or interfere with collagen crosslinking. Examples of genetic engineering approaches including, inserting a gene for the desired collagen, "knocking-out" the lysyl oxidase gene locus, downregulating lysyl oxidase with gene expression anti-sense strategies, changing the lysine residues in the telopeptides of the collagen molecule to different amino acids which are not substrates for lysyl oxidase, and altering cysteine residues involved in disulfide crosslinking.

Similarly, this approach will allow for elastin to be produced *in vitro* and harvested from cultured cells for use in biomedical applications, since tropoelastin (monomeric elastin) forms crosslinks in a manner analogous to collagens, requiring oxidation of specific lysine groups by lysyl oxidase. Therefore, elastin may be isolated and purified from the cell culture system of this invention.

Other proteins which are co-localized with collagens in the pericellular matrix around the cells may be harvested with the non-crosslinked collagens, for example, proteoglycans, such as decorin and biglycan, or glycoproteins, such as vitronectin, tenascin, fibronectin, and thrombospondin I. These molecules may then be isolated, purified, concentrated and used in biomedical applications.

The collagen-producing cells are grown or incubated under conditions to promote the growth of the cells in either media supplemented with serum or in chemically defined media (in other words, free of undefined animal organ or tissue extracts, for example, serum, pituitary extract, hypothalamic extract, placental extract or embryo extract). To the culture medium an agent to interfere with collagen crosslinking is added to inhibit or markedly reduce the formation of intra- and inter-molecular crosslinks. The culture medium that is used and the specific culturing conditions needed to promote cell growth and viability will depend on the type of cell being grown. The medium necessary to culture the cells needs to be a balanced medium, which may either contain a serum supplement (e.g. fetal or new born calf serum) such as Dulbecco's modified Eagle's medium, for others see Ham and McKeehan, *Methods in Enzymology,* 58:44-93 (1979), or a chemically defined medium, such as given in Example 1, for other appropriate chemically defined media see Bottenstein *et al.*, *Methods in Enzymology*, 58:94-109 (1979). To promote collagen synthesis the medium needs to be supplemented with sodium ascorbate or one of its more chemically stable derivatives e.g. L-ascorbic acid phosphate magnesium salt n-hydrate. Tsao and Young, *In Vitro Cellular and Developmental Biology* 31: 87-90 (1995).

To accomplish the synthesis of collagen by cells, without crosslinks, pharmacological agents may be added to the culture medium which affect either collagen crosslinking or collagen packing in the pericellular matrix. One group of such agents to interfere with collagen crosslinking are lysyl oxidase inhibitors. The preferred lysyl oxidase inhibitor is ß-aminopropionitrile. The invention, however, is not intended to be limited to this one specific lysyl oxidase inhibitor. Other lysyl oxidase inhibitors which can be

used in this invention include, but are not limited to, 2-bromoethylamine hydrobromide, *cis*-1,2-diaminocyclohexane, *trans*-2-phenylcyclopropylamine hydrochloride (tranylcypromine), 2-nitroethylamine hydrochloride, and 2-chloroethylamine hydrochloride. A further example of a pharmacological agent to interfere with collagen crosslinking is D-penicillamine, which inhibits crosslink formation by affecting the ability of the oxidized lysine residues to form covalent bonds with one another Nimni *et al.*, in "*Chemistry and Molecular Biology of the Intercellular Matrix*" (Balazs ed) Academic Press, New York, NY, 1970, pages 417-430.

Once the culture has ceased logarithmic growth, the medium is supplemented with an ascorbate to promote hydroxylation and secretion of procollagens. This stage of the culture is termed the biosynthetic phase. After an appropriate period of time in which the cells are secreting collagen, typically from about two days to about seven days, the conditions are changed to solubilize the collagen. In one embodiment, the medium is removed from the culture. In all embodiments, the cell layer is washed with a cold dilute neutral salt solution in order to dissolve and remove the synthesized non-crosslinked collagen. The dilute neutral salt solution needs to remain cold in order to prevent fibril formation of collagen. The preferred temperature range for the wash step is slightly above freezing, from about 2° to about 8°C. A requirement of this invention is that viable cells remain in the cell culturing system to resume synthesis of non-crosslinked collagen. Other wash protocols could therefore be used that would solubilize collagen but also permit cell survival. The cold dilute neutral salt solution, which at this point will contain the dissolved, non-crosslinked collagen, is then removed from the cell culture and fresh cell culture medium is placed on the same collagen-producing cell culture. In a preferred embodiment, after a period of time, typically from about two days to about seven days, the cold dilute neutral salt solution washing is repeated, and again, fresh cell culture medium is placed on the culture once the cold dilute neutral salt wash is removed. This cycling of cell culture medium with cold dilute neutral salt solution may be repeated a number of times.

The preferred agent to solubilize non-crosslinked collagens from the cell layer is Dulbecco's phosphate-buffered saline as supplied by BioWhittaker (Walkersville, MD). The invention, however, need not be limited to Dulbecco's phosphate-buffered saline, but may include Hanks' balanced salt solution and Earle's balanced salt solution. One of skill in the art would be able to formulate other solutions that would both solubilize collagen and allow for cell survival.

The washing times with the salt solution may vary between 30 minutes and six hours, with the longer time in some circumstances yielding more material. Similarly, the extent of agitation during the cold salt solution washes may affect the yield. Moreover, changes in the wash temperature from the preferred range of 2°C to 8°C may also yield more collagens or affect the recovery of other matrix components.

The non-crosslinked collagen may also be crosslinked by other chemical crosslinking agents, among those frequently used for collagenous based materials include gluteraldehyde, formaldehyde, carbodiimides, hexamethylene diisocyanate, bisimidates, glyoxal, polyglycerol polyglycidyl ether, adipyl chloride, dehydrothermal, UV irradiation and sugar-mediated, however, chemical crosslinking agents need not be limited to these examples as other crosslinking agents and methods known to those skilled in the art may be used.

This invention is thus also directed to non-crosslinked collagen. The preferred non-crosslinked collagen I contains no propeptides (the N- and C- propeptides), contains telopeptides, has greater than 40% of the total translated proline residues within the triple-helical region of the molecule hydroxylated, and contains two alpha1(I)-chain and one alpha2(I)-chains. Another preferred non-crosslinked collagen I contains no propeptides (the N- and C-propeptides), has no telopeptides, has greater than 40% of the total translated proline residues within the triple-helical region of the molecule hydroxylated, and contains two alpha1(I)-chain and one alpha2(I)-chains. In the preferred embodiment, the non crosslinked collagen is human collagen, and in the most preferred embodiment is human collagen I.

The non-crosslinked collagens may also be recovered from the cold dilute neutral salt solution wash by a variety of methods, such as by differential salt precipitation Trelstad R.L., "*Immunochemistry of the Extracellular Matrix,*" (Furthmayr ed.) CRC Press 1982, Volume 1, pages 31-42; Kielty *et al.*, in "*Connective Tissue and Its Heritable Disorders: Molecular, Genetic and Medical Aspects*" (Royce and Steinmann,eds.). Wiley-Liss, 1993, pages 103-147. dialysis, or by traditional tangential flow concentration using an ultrafiltration unit. In the preferred method, the salt-soluble collagens harvested in the cold salt solution may be concentrated by the use of an ultrafiltration unit with a nominal 100,000 kDa molecular weight cut-off.

Further, procollagens may be harvested from the spent cell culture medium by a variety of methods. Any method which provides for efficient removal of the procollagens from the culture medium and purification of the procollagens may be employed. Such approaches may include, treating the spent cell culture medium with pepsin and then performing differential salt precipitation, such that atelopeptide collagens are recovered. Alternatively, the spent cell culture medium may be passed over an affinity chromatography bed, with an antibody directed to one of the procollagen propeptides, and the procollagen collected. The thus collected procollagen may then be treated with the specific C- and N-proteinases to cleave the propeptides leaving intact fully processed, telopeptide containing collagen. The cleaved propeptides may be isolated and used therapeutically to treat disorders that involve an imbalance in collagen metabolism.

The cycling steps may be repeated until either the yield of extracted collagen from the cell layer ceases or the culture deteriorates. At this time, the collagens deposited in the cell layer which are not soluble in a dilute salt solution, may be solubilized by extracting the culture into dilute acetic acid and collected by differential salt precipitation. These are fully processed, telopeptide containing collagens, which have been crosslinked into the pericellular matrix.

EXAMPLES

The invention will be further described with reference to a specific embodiment using human fibroblast cells cultured in medium containing the lysyl oxidase inhibitor, ß-aminopropionitrile, dissolving the synthesized procollagen with a phosphate-buffered salt solution, and recovering and concentrating the procollagen using a hollow fiber. One of skill in the art will be able to choose other cells that naturally produce collagen, or can be genetically engineered to produce collagen, as well as alternative collagen crosslinking inhibitors and various salt solutions. The parameters and ranges of culture medium and culturing conditions is within the ability of one of skill in the art to select the various parameters and ranges and to test them to determine the operability to recover collagen as defined by the claims.

Example 1: Production of human collagen by fibroblasts in culture in the presence of a lysyl oxidase inhibitor subjected to routine extractions of human collagen from the cell layer by Dulbecco's phosphate-buffered saline.

Human foreskin fibroblasts (designated HDF B116, originated at Organogenesis Inc., Canton, MA) were serially passaged for the purposes of scaling up. The culture medium throughout the experiment was antibiotic-free and serum-free. Scale-up medium contained: Dulbecco's modified Eagle's medium (high glucose formulation)/Ham's F-12 medium (3:1, v/v) supplemented with GlutaMAX-I (Gibco BRL, Gaithersburg, MD) [$4x10^{-3}M$], hydrocortisone [0.4µg/ml], bovine insulin [5µg/ml], 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$], human transferrin [5µg/ml], ethanolamine [$1x10^{-4}M$], *ortho*-phosphorylethanolamine [$1x10^{-4}M$], calcium chloride [$1x10^{-3}M$], adenine [$1.8x10^{-4}M$], selenious acid [$5.3x10^{-8}M$], and murine epidermal growth factor [10ng/ml].

A sterilized, silconized (Prosil-28, PCR Inc., Gainesville, FL) 3L spinner flask (Bellco, Vineland, NJ), with 50g glass-coated

plastic beads (SoloHill, Ann Arbor, MI) was inoculated with $1x10^8$ cells in 1L of defined growth medium, composed of: Dulbecco's modified Eagle's medium (high glucose formulation)/Ham's F-12 medium (3:1, v/v) supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml], bovine insulin [5µg/ml], 3,3',5-triiodo-L-thyronine [$2x10^{-11}$M], human transferrin [5µg/ml], ethanolamine [$1x10^{-4}$M], *ortho*-phosphorylethanolamine [$1x10^{-4}$M], calcium chloride [$1x10^{-3}$M], adenine [$1.8x10^{-4}$M], selenious acid [$5.3x10^{-8}$M], murine epidermal growth factor [10ng/ml] and beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI). The flask was then placed in an incubator at 37±0.5°C/10±0.5% carbon dioxide. Cells were allowed to attach to the beads for 24 hours while the spinner was set at an intermittent cycle of 1 minute on/30 minutes off at 15-18 rpm. After attachment, the volume of growth medium was increased incrementally over several days to a final volume of 3L. The speed of the impeller was then set at 25 rpm, after 6 weeks the impeller speed was reduced to 21 rpm. Medium was replenished every 2-3 days by removing 2L of spent growth medium, leaving 1L of medium in the spinner flask, and adding 2L of freshly prepared growth medium. After 12 days, the culture had become confluent (i.e. the point at which the cells had covered the surface of the beads) and the growth medium was removed and replaced with production medium, which contained Dulbecco's modified Eagle's medium (high glucose formulation)/Ham's F-12 medium (3:1, v/v) supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 µg/ml], bovine insulin [5µg/ml], 3,3',5-triiodo-L-thyronine [$2x10^{-11}$M], human transferrin [5µg/ml], ethanolamine [$1x10^{-4}$M], ortho-phosphorylethanolamine [$1x10^{-4}$M], calcium chloride [$1x10^{-3}$M], beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI), L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Pure Chemical Industries, Ltd., Richmond, VA) [50µg/ml], L-proline [$1.93x10^{-3}$M] and glycine [$1.67x10^{-3}$M].

Cold cycling was implemented after two days in production medium. Spent production medium was removed from the vessel and 500ml of Dulbecco's phosphate buffered saline (BioWhittaker Inc.,

Walkersville, MD) at room temperature was added for one minute to dilute and remove any residual spent medium. The Dulbecco's phosphate buffered saline was then removed immediately from the spinner flask. To solubilize pericellular collagens, 1L of Dulbecco's phosphate buffered saline at 4-8°C was added to the vessel, which was placed in a refrigerator for one hour at 4-8°C and the vessel manually agitated every ten minutes. The Dulbecco's phosphate buffered saline, containing collagens solubilized from the cell layer, was removed and fresh production medium at 37°C was added to the vessel. The vessel was returned to the incubator at 37±0.5°C/10±0.5% carbon dioxide. The culture was maintained for 133 days after the introduction of production medium to the cell culture system. Cycling was repeated every 2-3 days throughout the 133 days. The Dulbecco's phosphate buffered saline from the cold cycling was kept frozen at -20°C until concentrated.

To concentrate the solubilized collagens, 22L of Dulbecco's phosphate buffered saline containing collagens from the cell layer from the first 22 cold cycles was thawed overnight at between 4-8°C. Once thawed, the Dulbecco's phosphate buffered saline was acidified with acetic acid to give a final concentration 0.05% (v/v). The acidified mixture was then concentrated using a hollow fiber cartridge with a 100,000 molecular weight cut-off (A/G Technologies, Needham, MA) in a closed loop that allowed the removal of molecules less than the molecular weight cut-off. The final volume of the concentrate was 368 ml.

Example 2: Separation and identification of collagen alpha-chains extracted from the cell layer with Dulbecco's phosphate-buffered saline from the individual cold cycles by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Aliquots of the Dulbecco's phosphate buffered saline from each cold cycle were taken and denatured in sodium dodecyl sulfate (0.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v)

at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a tris/glycine/sodium dodecyl sulfate buffer system at 120V (constant voltage) for 2-3 hours (Laemmli, Nature, 1970, 227, 680-685). The gels were stained with Page Blue 83 (0.1% w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gels revealed that each individual sample of the human collagen extracted from the cell layer with Dulbecco's phosphate-buffered saline at 4-8°C, contained bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gels. No other bands were revealed by Page Blue 83 staining on the gels at the loadings used.

Example 3: Separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the concentrated human collagen extracted from the cell layer with Dulbecco's phosphate-buffered saline.

To identify the major collagenous species present in the collagen preparation the material was subjected to pepsin, to cleave the telopeptides and propeptides, and then subjected to polyacrylamide gel electrophoresis. To compare with the concentrated human collagen extracted from the cell layer with Dulbecco's phosphate-buffered saline, bovine collagen which was acid extracted from calf tendon (U.S. 5,106,949; Kemp et al, Organogenesis Inc., Canton, MA) and pepsin extracted human collagen I (Sigma type VIII collagen, Sigma Chemical Co., St. Louis, MO) were dissolved at 1.0±0.1mg/ml. Samples of the different collagens were digested by pepsin (100µg/ml) in aqueous acetic acid (0.5M, final concentration) at 4°C for 16 hours (Lamande & Bateman, Matrix 1993, 13, 323-330).

Samples of both the pepsin treated collagen and non-pepsin treated collagens were then prepared for separation by polyacrylamide gel electrophoresis. Samples of the collagens were

denatured at 100°C for three minutes in the presence of sodium dodecyl sulfate (0.2%, w/v) and glycerol (8%, w/v). Samples were separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a tris/glycine/sodium dodecyl sulphate buffer system at 120V (constant voltage) for 2.5 hours (Laemmli, Nature, 1970, 227, 680-685). The gels were stained with Page Blue 83 (0.1%, w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for one hour. Diffusional destaining of the gel was performed with aqueous acetic acid (8%, v/v) for 60 hours and the gel dried. The gel, shown in Figure 1, revealed that the majority of the material extracted in the Dulbecco's phosphate-buffered saline ran as collagen alpha1(I)- and alpha2(I)-chains. From comparison of the pepsin treated material with the non-pepsin treated material the other bands are most likely to be [alpha1(III)]3, and procollagen I species. Most obvious from the gel is the lack of beta components of collagen I (Sokolov et al, Medical Science Research, 1989, 17, 911-913). The lack of the material at the very top of the gel, further suggests that the material extracted by the Dulbecco's phosphate buffered saline is monomeric and contains few intermolecular crosslinks.

Example 4: Quantification of collagen in the individual extractions of human collagen from the cell layer with Dulbecco's phosphate buffered saline.

Collagen is frequently quantified based on the amount of hydroxyproline present in the material. Hydroxyproline may be measured by a specific chemical assay (Woessner, Arch Biochem Biophys, 1961, 93, 440-447) described in detail below.

Aliquots of the Dulbecco's phosphate buffered saline (1ml) from each cold cycle were taken and hydrolyzed in an equal volume of hydrochloric acid (1ml, 12M) at 110°C for 16 hours. The hydrolysate was then neutralized with sodium hydroxide (10M). Samples were taken from the neutralized hydrolysate (100µl) and applied to wells on a 96-well microplate. Amino acid standards

(Collagen Hydrolyzate #A9531, Sigma Chemical Co, St Louis, MO) at various concentrations to generate a standard curve were also applied to the same 96-well plates as the samples. To each well chloramine-T (50µl, 0.05M (Mallinckrodt Chemicals, Chesterfield, MO) in water/ethylene glycol monomethyl ether/acetate buffer pH6 (2:3:5 v/v) was added and allowed to incubate at room temperature for 20 minutes. After incubation, perchloric acid (50µl, 3.15M; J.T. Baker, Phillipsburg NJ) was added to each well and allowed to incubate for 5 minutes at room temperature. Then *para*-dimethylaminobenzaldehyde (Aldrich Chemical Co., Milwaukee, WI) dissolved in ethylene glycol monomethyl ether (50µl, 20% , w/v) was added to each well and the plate was incubated at 60°C for 20 minutes. Immediately after incubation, the plates were read using a microplate spectrophotometer at 570 nm. A standard curve was plotted of absorbance at 570nm versus the amount of hydroxyproline based on the serial dilutions of the amino acid standards. The slope of this curve was used to calculate the concentration of hydroxyproline in each sample. Collagen concentration was calculated assuming that collagen contains 12.2% (w/w) hydroxyproline (Laurent et al, Anal Biochem, 1981, 113, 301-312). The results, shown in Figure 2, demonstrate that collagen was solubilized from the cell layer with Dulbecco's phosphate-buffered saline in every cold cycle.

Example 5: Quantification of collagen in the concentrated extract of human collagen from the cell layer with Dulbecco's phosphate-buffered saline.

Aliquots of the acidified concentrated collagen (1ml) were taken and hydrolyzed in an equal volume of hydrochloric acid (1ml, 12M) at 110°C for 16 hours. Samples were cooled and then neutralized with the addition of sodium hydroxide (1ml, 10M). The samples were then diluted to 200ml with water. Three samples of the diluted solution (1ml) and a series of amino acid standards (Collagen Hydrolyzate #A9531, Sigma Chemical Co, St Louis, MO)

were assayed. To each sample and standard chloramine-T (0.5ml, 0.05M (Mallinckrodt Chemicals, Chesterfield, MO) in water/ethylene glycol monomethyl ether/acetate buffer pH6 (2:3:5 v/v)) was added, the solution was mixed and allowed to incubate at room temperature for 20 minutes (Woessner, Arch Biochem Biophys 1961, 93, 440-447). Perchloric acid (0.5ml, 3.15M; J.T. Baker, Phillipsburg NJ) was then added to each tube. Samples were mixed and incubated at room temperature for five minutes. *Para*-Dimethylaminobenzaldehyde (0.5ml, 20% w/v in ethylene glycol monomethylether, Aldrich Chemical Co., Milwaukee, WI) was then added and mixed. Samples were then incubated at 60°C for 20 minutes. After incubation, samples were allowed to cool and the absorbance was read at 560nm in a spectrophotometer. A standard curve was plotted of absorbance at 560nm versus the amount of hydroxyproline based on the serial dilutions of the amino acid standards. The slope of this curve was used to calculate the concentration of hydroxyproline in each sample. Collagen concentration was calculated assuming that collagen contains 12.2% (w/w) hydroxyproline. Laurent *et al*, Analytical Biochemistry, 113:301-312 (1981). The amount of human collagen recovered from the cell layer by Dulbecco's phosphate-buffered saline extractions and concentrated by hollowfiber dialysis was between 330-350 mg. The final collagen concentration of the material was 0.90-0.95 mg/ml.

Example 6: Partial amino acid analysis of the concentrated human collagen extracted from the cell layer with Dulbecco's phosphate-buffered saline.

For amino acid analysis of samples Waters Pico-Tag System was used (Bidlinger et al, Journal of Chromatography, 1984, 336, 93-104) as described in the Pico-Tag method handbook (Millipore Corp., Bedford, MA). Briefly, samples of the concentrated human collagen extracted from the cell layer with Dulbecco's phosphate buffered saline, bovine collagen which was acid extracted from calf tendon (U.S. 5,106,949; Kemp et al, Organogenesis Inc., Canton, MA)

and pepsin extracted human collagen I (Sigma type VIII collagen, Sigma Chemical Co., St. Louis, MO) were dissolved at 1.0±0.1mg/ml. Aliquots in triplicate (0.1ml) were lyophilized and hydrolyzed in the vapor phase with hydrochloric acid (6M) containing phenol (1%, w/v) under nitrogen at 145°C for one hour. The samples were resuspended in ethanol:water:triethylamine (2:2:1 v/v) and lyophilized. The samples were then derivatized with phenylisothiocyanate (Pierce Chemical Co., Rockford, IL):ethanol:water:triethylamine (1:7:1:1 v/v) at room temperature for 20 minutes and then frozen. The samples were then lyophilized and prepared for high performance liquid chromatography by resuspending the residues in Pico-Tag Sample Diluent (Millipore Corporation, Bedford, MA). Derivatized samples were injected on to a Waters 150mmx3.9mm reverse-phase C18 column (Millipore Corporation, Bedford, MA) and the derivatized amino acids were eluted with a sodium acetate-acetonitrile gradient (Pico-Tag eluents A and B, Millipore Corporation, Bedford, MA). Peak areas for representative peaks were determined by integration and comparison with standards. The results are shown in Table 2. The results indicate that the major amino acids are present in the human collagen extracted from the cell layer at approximately the correct prevalence, when compared with other human and bovine collagens.

Table 2
Comparison of the Partial Amino Acid Composition of Collagens

Amino acid	Residues/1000			
	Literature Human Collagen I	Cell layer Human Collagen	Sigma Human Collagen I (Pepsin)	Bovine
Hyp	103	97	99	100
Gly	335	339	379	322
Ala	111	101	95	94
Pro	120	116	107	119

Literature human collagen I values obtained from Nimni and Harkness, in *Collagen*, "Biochemistry," Volume I (Nimni ME ed) 1988, pages 1-77. Cell layer human collagen was extracted by Dulbecco's phosphate-buffered saline and concentrated as described in Example 1. Bovine collagen is Organogenesis Inc.'s lot#206.

Example 7: Production of human collagen by fibroblasts in culture: Continuation of Example 1.

This Example is a continuation of Example 1.

Cold cycling of the 3L spinner flask (Bellco, Vineland, NJ.) culture from Example 1 was continued, under the same conditions,

for a further 42 days. Thus, the culture was maintained in collagen production medium for a total of 175 days, including 73 collagen solubilizations with 4-8°C Dulbecco's phosphate buffered saline with magnesium and calcium (BioWhittaker Inc., Walkersville, MD.). The Dulbecco's phosphate buffered saline from the cold cycling was kept frozen at -20°C until concentrated.

To concentrate the solubilized collagens, 51L of Dulbecco's phosphate buffered saline containing collagens from the cell layer from cold cycle number 23 through cold cycle number 73 were thawed overnight at between 4-8°C. Once thawed, the Dulbecco's phosphate buffered saline was acidified with acetic acid to give a final concentration 0.05% (v/v). The acidified mixture was then concentrated using a hollow fiber cartridge with a 100,000 molecular weight cut-off (A/G Technologies, Needham, MA) in a closed loop that allowed the removal of molecules less than the molecular weight cut-off. The final volume of the concentrate was 315ml.

The collagen was quantified based on the amount of hydroxyproline in a sample of the concentrated human collagen, as described below. Aliquots of the acidified concentrated collagen (1ml) were taken and hydrolyzed in an equal volume of hydrochloric acid (1ml, 12M) at 110°C for 16 hours. The hydrolysate was then neutralized with sodium hydroxide (10M). Samples were taken from the neutralized hydrolysate (100µl) and applied to wells on a 96-well microplate. Amino acid standards (Collagen Hydrolyzate #A9531, Sigma Chemical Co, St Louis, MO) at various concentrations to generate a standard curve were also applied to the same 96-well plates as the samples. To each well chloramine-T (50µl, 0.05M (Mallinckrodt Chemicals, Chesterfield, MO) in water/ethylene glycol monomethyl ether/acetate buffer pH6 (2:3:5 v/v) was added and allowed to incubate at room temperature for 20 minutes. After incubation, perchloric acid (50µl, 3.15M; J.T. Baker, Phillipsburg NJ) was added to each well and allowed to incubate for 5 minutes at room temperature. Then *para*-dimethylaminobenzaldehyde (Aldrich Chemical Co., Milwaukee, WI) dissolved in ethylene glycol monomethyl ether (50µl, 20% , w/v) was added to each well and the

plate was incubated at 60°C for 20 minutes. Immediately after incubation, the plates were read using a microplate spectrophotometer at 570 nm. A standard curve was plotted of absorbance at 570nm versus the amount of hydroxyproline based on the serial dilutions of the amino acid standards. The slope of this curve was used to calculate the concentration of hydroxyproline in each sample. Collagen concentration was calculated assuming that collagen contains 12.2% (w/w) hydroxyproline. Laurent *et al*, *Analytical Biochemistry* 113:301-312 (1981). The amount of human collagen recovered from the cell layer by Dulbecco's phosphate buffered saline extractions and concentrated by hollowfiber dialysis was 463mg. The final collagen concentration of the material was 1.47mg/ml.

Example 8: Production of human collagen by fibroblasts cultured in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of human collagen from the cell layer by Dulbecco's phosphate-buffered saline.

Human neonatal foreskin fibroblasts (designated HDF B119, originated at Organogenesis Inc. Canton, MA) were cultured and serially passaged for the purposes of scaling-up. The culture medium throughout the experiment was antibiotic-free and serum-free. Scale-up medium contained: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}M$] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Co., St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$] (Sigma Chemical Co., St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), ethanolamine [$1x10^{-4}M$] (Sigma Chemical Co., St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}M$] (Sigma

Chemical Co., St. Louis, MO), adenine [1.8x10^{-4}M] (Sigma Chemical Co., St. Louis, MO), selenious acid [5.3x10^{-8}M] (Aldrich Chemical Co., Milwaukee, WI), and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY).

A sterilized, silconized (Prosil-28, PCR Inc., Gainesville, FL) 3L spinner flask (Bellco, Vineland, NJ), with 50g glass-coated plastic microcarriers (SoloHill, Ann Arbor, MI) was inoculated with 2.6X10^{8} cells in 1L of defined growth medium, composed of: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L- glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [4x10^{-3}M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Co., St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), 3,3',5-triiodo-L-thyronine [2x10^{-11}M] (Sigma Chemical Co., St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), ethanolamine [1x10^{-4}M] (Sigma Chemical Co., St. Louis, MO), *ortho*-phosphorylethanolamine [1x10^{-4}M] (Sigma Chemical Co., St. Louis, MO), adenine [1.8x10^{-4}M] (Sigma Chemical Co., St. Louis, MO), selenious acid [5.3x10^{-8}M] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY). The flask was then placed in an incubator maintained at a temperature of 37.0±0.5°C with an atmosphere enriched with 10.0±0.5% carbon dioxide. Cells were allowed to attach to the beads for 24 hours while the spinner was set at an intermittent cycle of 1 minute on/30 minutes off at 15-20 r.p.m. After attachment, the volume of growth medium was increased incrementally over several days to a final volume of 3L. The speed of the impeller was then set at 18-22 r.p.m. Medium was replenished every 2-3 days by removing 2L of spent growth medium, leaving 1L of medium in the spinner flask, and adding 2L of freshly prepared growth medium. After 10 days, the culture had become confluent (i.e. the point at which the cells had covered the surface of the microcarriers) and the microcarriers had started to clump. At this time the growth medium was removed and replaced with collagen production medium, which

contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}M$] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 µg/ml] (Sigma Chemical Co., St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$] (Sigma Chemical Co., St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), ethanolamine [$1x10^{-4}M$] (Sigma Chemical Co., St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}M$] (Sigma Chemical Co., St. Louis, MO), selenious acid [$5.3x10^{-8}M$] (Aldrich Chemical Co., Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI), L-ascorbic acid phosphate magnesium salt n-hydrate [50µg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}M$, final concentration] (Sigma Chemical Co., St. Louis, MO) and glycine [$1.67x10^{-3}M$, final concentration] (Sigma Chemical Co., St. Louis, MO).

Cold cycling was implemented to harvest collagen from the cell layer after two days in collagen production medium. Spent collagen production medium was removed from the vessel and 500 ml of Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) at room temperature was added for one minute to dilute and remove any residual spent collagen production medium. The Dulbecco's phosphate buffered saline was then removed immediately from the spinner flask. To solubilize the pericellular collagens, 1L of Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) at 4-8°C was added to the vessel, which was placed in a refrigerator for one hour at 4-8°C and the vessel was manually agitated every ten minutes. The Dulbecco's phosphate buffered saline, containing collagens solubilized from the cell layer, was removed and fresh collagen production medium at 37°C was added to the vessel. The vessel was returned to the incubator maintained at a temperature of 37.0±0.5°C with an atmosphere enriched in carbon dioxide

(10.0±0.5%). Cycling was repeated every 2-3 days. Through 150 days, 57 liters of Dulbecco's phosphate buffered saline containing solubilized collagen had been harvested. The Dulbecco's phosphate buffered saline from the cold cycling was kept frozen at -20°C until concentrated, as described below. As of day 157 in collagen production medium the culture was still being maintained.

To concentrate the solubilized collagens, 57L of Dulbecco's phosphate buffered saline with calcium and magnesium, containing collagens harvested from the cell layer, from the first 57 cold cycles was thawed at between 4-8°C. Once thawed the solutions were combined in a single vessel. From that vessel the solution was pumped through a stainless steel strainer and a five micron clarification filter (PALL Corporation, East Hills, New York) to remove any glass-coated plastic microcarriers and cellular debris remaining in the harvested samples. Following this, the Dulbecco's phosphate buffered saline containing the solubilized collagens was acidified with glacial acetic acid (J.T. Baker, Inc., Phillipsburg, NJ) to give a final concentration of acetic acid of 0.05% (v/v). The acidified solution containing the solubilized collagens was then concentrated to approximately three liters using a hollow fiber-type ultrafiltration cartridge with a 100,000 molecular weight cut-off (310mm (length) x 32mm (diameter); A/G Technologies, Needham, MA) in a closed system. The ultrafiltration cartridge allows for the removal of material smaller than the molecular weight cut-off. Following this, the solution was diafiltered in the same ultrafiltration system against five volumes of aqueous acetic acid (0.05%, v/v) to remove any residual salts and media components, so that the collagen is in a aqueous acetic acid solution. The collagen was then further concentrated in the same system to a final volume of 450 ml.

To identify the concentrated material as human collagen, it was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis to look for characteristic collagen alpha-chains. A sample of the concentrated human collagen (42µl) was denatured in sodium dodecyl sulfate (0.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v), final volume 100µl, at 100°C for three

minutes. A sample of the concentrated human collagen from Example 7 was similarly prepared. These samples were then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, w/v) buffer system at 20 amps (constant current) for 1.9 hours. Laemmli, *Nature*, 227: 680-685 (1970). The gels were stained with Page Blue 83 (0.1%, w/v; Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1.5 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 36 hours. The gel, shown in figure 3, revealed that the concentrated sample of the human collagen extracted from the cell layer with Dulbecco's phosphate-buffered saline at 4-8°C, contained bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gel.

Example 9: Fabrication of a dense fibrillar collagen (DFC) construct.

A transwell (24mm, k-resin, 3.0μm pore size; Costar Corporation, Cambridge, MA) was situated on top of a 150ml glass beaker, such that the membrane of the transwell was approximately 1cm below the top of the beaker. The beaker was then filled to the height of the membrane surface with polyethylene glycol (20%, w/v, molecular weight 8,000; Spectrum Chemical Manufacturing Company, Gardena, CA) in a sodium phosphate buffer (pH 6.83; Na_2HPO_4, 2.84%, w/v, J.T.Baker Company, Phillipsburg, NJ; $NaH_2PO_4 \cdot H20$, 3.11%, w/v, J.T. Baker Company, Phillipsburg, NJ; NaCl, 0.49%, w/v, Sigma Chemical Company, St. Louis, MO). A stirrer bar was placed into the beaker and the beaker was then situated on a magnetic stirrer plate (Scientific Products, McGaw, IL) which was placed in a 4-8°C chamber. Concentrated human collagen from Example 1 (4.0ml; 0.95mg/ml) was added to the transwell and the polyethylene glycol solution (see above) was gently stirred. Saran Wrap® was then placed over the beaker and transwell to minimize evaporation of the collagen solution and to prevent contamination by particulate matter. Additional volumes of concentrated human collagen solution

were added at two hour intervals such that a total of 8.0ml of concentrated human collagen was added to the transwell. The collagen was allowed to deposit onto the transwell membrane for a total of 36 hours. The transwell membrane was then taken out of the polyethylene glycol solution (see above) and placed in a dehydration chamber with a relative humidity of 20% at 4-8°C for 21 hours.

The DFC construct was then rehydrated by placing the transwell containing the dehydrated DFC construct into a 150mL glass beaker, which was filled with deionized water so that the dehydrated DFC construct was wholly submerged. A stir bar was added to the beaker and the beaker was placed on stirring plate. The deionized water was slowly stirred to facilitate the rehydration of the DFC and to rinse off any residual polyethylene glycol. The construct was rehydrated for 15 minutes and then the water was drained from the construct. The construct was then again placed into the dehydration chamber for 72 hours. The DFC construct was then rehydrated as before for a period of one hour.

The DFC construct was prepared for transmission electron microscopy, as described below. The DFC construct was fixed for 48 hours in paraformaldehyde (2%, v/v), glutaraldehyde (2.5%, v/v) and acrolein (1%, v/v) in sodium cacodylate (0.1M, pH7.4). The sample was postfixed in osmium tetroxide (1%, w/v) in sodium cacodylate (0.1M, pH7.4) and stained en bloc with aqueous uranyl acetate (2%, w/v). After secondary fixation, the sample was dehydrated in a graded ethanol series and propylene oxide, and embedded in Epox-812 (Ernest F. Fullam, Rochester, NY). Ultrathin sections (about 700nm) were stained with uranyl acetate and lead citrate, and examined using a Joel JEM 100S transmission electron microscope at 80kV. The electron micrographs showed large numbers of aggregates of banded collagen fibrils, see figure 4. The longitudinal cross-sections of the aggregates had a honeycomb appearance, with large 'holes' between groups of fibrils. Some areas contained finer fibrils, which had not aggregated to form fibers, which look like 'teased' aggregates. The D-period banding was measured and was 58.9±1.7nm (n=10). This figure is within the acceptable ranges for collagen I D-

periods, which have been reported to be 67nm *in vivo* and 60nm after dehydration. Parry and Craig, *Bioploymers* 16:1015-1031 (1977).

Example 10: Fabrication of collagen sponge from human collagen

Concentrated human collagen from Example 1 (5ml, 0.95mg/ml) from cold Dulbecco's phosphate buffered saline wash cycles was added to a polystyrene tissue culture dish (35mmx10mm; Costar Corporation, Cambridge, MA). A cover was placed over the dish and secured with tape. The dish was then placed into a styrofoam container such that the culture dish was situated in the middle of the container space. The collagen solution was allowed to freeze slowly. This was accomplished by first freezing the collagen solution at -20°C for sixteen hours and then at -80°C for sixteen hours. The cover of the dish was removed and the dish with the frozen collagen solution was placed into a lyophilizer vial (1200 mL volume; VirTis Co., Gardiner, NY) and placed onto a lyophilizer (VirTis Co., Gardiner, NY) until the collagen solution was fully lyophilized. The lyophilized collagen was then redissolved in a minimum of acetic acid (2ml, 0.5M, J.T. Baker, Phillipsburg, NJ) with gentle mixing. To this human collagen in acetic acid was added 3ml of concentrated human collagen, from Example 1) and the process of freezing the human collagen in the cell culture dish followed by lyophilization and redissolution was repeated until a total of 25mg of human collagen was added to the cell culture dish. Once all 25mg of human collagen was thoroughly lyophilized, the resulting sponge was examined and photographed (see figure 5).

Example 11: Quantification of proteoglycan/ glycosaminoglycan content of concentrated human collagen extracted with Dulbecco's phosphate buffered saline from the cell layer of human neonatal foreskin fibroblasts

cultured in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile.

To determine the amount of proteoglycans and glycoaminoglycans present in the collagen preparation, a commercial assay kit for proteoglycans and glycosaminoglycans was used (Blyscan Proteoglycan and Glycosaminoglycan Assay kit, Biocolor Ltd., Belfast, Northern Ireland). This kit is based on the specific binding of proteoglycans and glycosaminoglycans to a dye, 1,9-dimethyl-methylene blue. The binding of proteoglycans and glycosaminoglycans to the dye forms an insoluble complex that precipitates out of aqueous solutions. This precipitated complex is pelleted by centrifugation. The supernatant, which contains any unbound dye, is decanted off and the pellet is then resuspended in an isopropanol/chaotropic salt solution. This liberates the dye from the dye-proteoglycan/glycosaminoglycan complex, which may then be quantified spectrophotometrically.

Aliqouts (100µl) of two human collagen samples from Example 1 (0.95mg/ml) and Example 7 (1.4mg/ml) in duplicate were placed in 1.5ml microfuge tubes for the determination of the proteoglycans and glycosaminoglycan content of the sample. A set of standards was also prepared in duplicate: 1.0,2.0,3.0,5.0 µg of chondroitin-4-sulfate purified from bovine trachea, as supplied in the kit. To all test samples and standards 1ml of Blyscan Dye Reagent (1,9-dimethyl-methylene blue) was added and the tubes were all vigorously mixed with a vortex mixer. The tubes were allowed to react at room temperature for 30 minutes, allowing the dye reagent to form an insoluble complex with any proteoglycans and glycosaminoglycans present. Samples and standards were then centrifuged in a Beckman high-speed centrifuge (Model J2-21, Beckman Instruments, Inc., Palo Alto, CA) using a JA-18.1 rotor at 8000g for 10 minutes. The supernatant from all tubes was then removed by decanting, and any remaining drops of supernatant in the tubes were removed by inverting the tubes and gently tapping them onto a paper towel, being careful not to disturb any pellet that may be present in the tubes. To all samples and standards the Blyscan

Dissociation Reagent (1.0ml) was added to redissolve any pellet and to release the bound dye into solution. Samples and standards were mixed using a vortex mixer for five seconds and allowed to stand at room temperature until any pellet was completely dissolved. Samples and standards were then aliquoted into cuvettes. The absorbances of both samples and standards were measured at 656nm using a DU-50 Beckman spectrophotometer (Beckman Instruments, Palo Alto, CA) using distilled water as a blank. The mean absorbance of each standard was then plotted versus the amount of chondroitin-4-sulfate (μg). The equation of the line was used to calculate the amount of proteoglycans and glycosaminoglycans present in both human collagen samples. The results demonstrated that both samples of human collagen, at between 1 and 1.5mg/ml, contained less than 1μg of proteoglycans and glycosaminoglycan per 100μl of human collagen. These results indicate that both samples of human collagen examined contain negligible amounts of proteoglycans and glycosaminoglycans in their preparations.

Example 12: Separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the cyanogen bromide-derived peptides of concentrated human collagen to demonstrate the presence of collagen III.

To determine whether collagen III is present in the concentrated human collagen samples, the collagens were digested with cyanogen bromide and mapped by polyacrylamide gel electrophoresis. Digestion of collagens with cyanogen bromide, which cleaves proteins at unoxidized methionine residues, reveals a discrete and recognizable peptide map. This approach is capable of distinguishing between collagen I and collagen III from each other in a number of different mammalian species. Laurent *et al.*, *Analytical Biochemistry*, 113: 3011-312 (1981); Kirk *et al.*, *Collagen and Related Research*, 4:169-182 (1984); Lillie *et al.*, *Methods in Enzymology*, 145: 171-183 (1987); Mays *et al.*, *Mechanisms of Aging and Development*, 45:203-212 (1988).

Aliquots of concentrated human collagen, from Example 1 were pipetted into 1.5ml microfuge tubes. Samples of pepsin extracted human collagen I (Sigma type VIII collagen, Sigma Chemical Company, St. Louis, MO) and pepsin extracted human collagen III (Sigma type VIII collagen, Sigma Chemical Company, St. Louis, MO) were also aliqouted into 1.5ml microfuge tubes All the tubes were covered with Parafilm® (American National Can, Neenah, WI) which was perforated and the tubes were frozen at -80°C for four hours and then lyophilized to dryness (VirTis Co. Gardiner, NY).

The lyophilized samples were resuspended in 0.8ml of 70% formic acid (v/v; EM Science, Gibbstown, NJ) which was purged with nitrogen gas to remove dissolved oxygen. Cyanogen bromide (Sigma Chemical Company, St. Louis, MO) was dissolved in 70% formic acid (v/v) to achieve a 50mg/ml solution with respect to cyanogen bromide, and was purged with nitrogen gas for three minutes. Aliquots of the cyanogen bromide solution (0.2ml) were added to the resuspended samples, giving a final concentration of 10mg/ml of cyanogen bromide. Samples were purged with nitrogen gas for five seconds. Tubes were capped and the digestion was allowed to proceed for 16 hours at room temperature. Samples were then aliquoted out (3x0.3ml) into 1.5ml microfuge tubes, frozen at -80°C for four hours and then lyophilized to dryness.

Freeze-dried aliquots were prepared for electrophoresis by resuspending digested samples in sodium dodecyl sulfate (3%, w/v) in Tris-Cl (0.06M, pH6.8) and heating at 40°C for three minutes. Samples were denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide gradient minigel (8-16%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, w.v) running buffer at 125V (constant voltage) for three hours. Laemmli, *Nature*, 227:680-685 (1970). The separation was performed under non-reducing conditions to avoid the migration of the disulfide crosslinked alpha1(III)CB9 peptide, which migrates close to alpha1(III)CB5 peptide when the samples are reduced. Kirk *et al.*, *supra*; Turner and Laurent, Biochemical Society Transactions 14:1079-1080, (1986). The gel was stained

with Page Blue 83 (0.1%, w/v; Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel shown in figure 6, demonstrated that the classic marker peptide for collagen III, alpha1(III)CB5 peptide fragment, was present in the samples, indicating the presence of collagen III in the human collagen preparation.

Example 13: Separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the cyanogen bromide-derived peptides of concentrated human collagen I to demonstrate the presence of telopeptides.

To demonstrate the presence of telopeptides on human collagen I the collagens were digested with cyanogen bromide and the peptides mapped by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The alpha1(I)CB6 peptide which arises from the carboxy-terminus of the alpha1(I)-chain includes the carboxy-telopeptide of the alpha1(I)-chain in non-pepsin treated samples. Chandrakasan *et al., Journal of Biological Chemistry* 251:6062-6067 (1976). In collagen samples with no telopeptides (e.g. pepsin-treated) the alpha1(I)CB6 peptide migrates as a lower molecular weight peptide, allowing the presence of telopeptides to be determined based on these two forms of the alpha1(I)CB6 peptide. [To distinguish between the two peptides, the peptide without the telopeptide has been designated alpha1(I)CB6'].

For pepsin digestion, aliquots of concentrated human collagen from Example 8 were pipetted into 1.5ml microfuge tubes (0.75ml). Pepsin (Sigma Chemical Company, St. Louis, MO; at 200µg/ml in 1M acetic acid) was added to each sample, such that the final concentration of pepsin was 100µg/ml in 0.5M acetic acid. Samples were digested for 16 hours at 4°C. Sample tubes were then frozen at -80°C for four hours and then lyophilized to dryness. These samples were then digested by cyanogen bromide as described below.

Samples of non-pepsin treated human collagen from Example 8, as well as collagen I standard, pepsin extracted from human placenta (Sigma type VIII collagen, Sigma Chemical Company, St. Louis, MO) and collagen III standard, pepsin extracted from human placenta (Southern Biotechnology Associates, Birmingham, AL), were aliquoted into 1.5ml microfuge tubes, covered with Parafilm®, which was then perforated, the tubes were frozen at -80°C for four hours and lyophilized to dryness. The lyophilized samples of non-pepsin treated collagen and pepsin-treated collagen, from above, were resuspended in 0.8ml of 70% formic acid (v/v; EM Science, Gibbstown, NJ) which was purged with nitrogen gas to remove any dissolved oxygen. Cyanogen bromide (Sigma Chemical Company, St. Louis, MO) was dissolved in 70% formic acid (v/v) to achieve a 50mg/ml solution with respect to cyanogen bromide, and was purged with nitrogen gas for three minutes. Aliquots of the cyanogen bromide solution (0.2ml) were added to the resuspended samples, giving a final concentration of 10mg/ml with respect to cyanogen bromide. Samples were purged with nitrogen gas for five seconds. Tubes were capped and the digestion was allowed to proceed for 16 hours at room temperature. Samples were then aliquoted out (2x0.45ml) into 1.5ml microfuge tubes, frozen at -80°C for four hours and then lyophilized to dryness.

Lyophilized aliquots of the cyanogen bromide digested collagens were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis by resuspending the samples in sodium dodecyl sulfate (3%, w/v) in Tris-Cl (0.06M, pH6.8) and heating at 60°C for five minutes. Samples were denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) at 100°C for three minutes and the peptides separated by electrophoresis in a polyacrylamide minigel (16%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, w/v) running buffer at 125V (constant voltage) for 2.5 hours. Laemmli, *Nature*, 227: 680-685 (1970). The gel was stained with Page Blue 83 (0.1%, w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel, shown in figure 7, demonstrated that the

collagen preparation contained intact telopeptides as judged by the presence of the alpha1(I)CB6 band in the non-pepsin treated sample of human collagen. In the non-pepsin treated human collagen sample some of the alpha1(I)CB6' peptide was also present, this probably arises due to partial degradation of the non-helical telopeptide region.

Example 14: Production of collagen by human fibroblasts, cell strain HDF B116, cultured in serum-free medium in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of human collagen from the cell layer by Dulbecco's phosphate-buffered saline.

Human neonatal foreskin fibroblasts (designated HDF B116, originated at Organogenesis Inc., Canton, MA) were seeded at $5x10^5$cells/75cm^2 in four plastic tissue culture-grade flask (T75, Costar Company, Cambridge, MA) in serum-free, antibiotic-free growth medium, as described below. The serum-free growth medium contained: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker, Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3', 5-triiodo-L-thyronine [$2x10^{-11}$M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), *ortho-* phosphorylethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), adenine [$1.8x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}$M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate

Biotechnology Inc., Lake Placid, NY). The cells were maintained in this growth medium in an incubator at a temperature of 37.0±0.5°C with an atmosphere of 10.0±0.5% carbon dioxide in air. The growth medium was replaced every 3-4 days with freshly prepared growth medium. After seven days the cells had become highly confluent, that is the cells had formed a densely packed layer at the bottom of the tissue culture flask. At this time the growth medium was removed and replaced with collagen production medium, which contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}M$] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 µg/ml] (Sigma Chemical Co., St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$] (Sigma Chemical Co., St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), ethanolamine [$1x10^{-4}M$] (Sigma Chemical Co., St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}M$] (Sigma Chemical Co., St. Louis, MO), selenious acid [$5.3x10^{-8}M$] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI), L-ascorbic acid phosphate magnesium salt n-hydrate [50µg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}M$, final concentration] (Sigma Chemical Co., St. Louis, MO) and glycine [$1.67x10^{-3}M$, final concentration] (Sigma Chemical Co., St. Louis, MO). The collagen production medium was replaced every 3-4 days with freshly prepared collagen production medium. After seven days cold cycling to harvest the collagen in Dulbecco's phosphate-buffered saline was performed. Spent collagen production medium was aspirated and cells were rinsed for one minute with 5ml of room temperature Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) to remove any residual medium. The Dulbecco's phosphate buffered saline was aspirated thereafter. To solubilize the pericellular collagens, 5ml of 4-8°C Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q;

BioWhittaker Inc., Walkersville, MD) was added to each flask and the flask was placed at 4-8°C for one hour. After one hour, the Dulbecco's phosphate buffered saline, containing collagens solubilized from the cell layer, was aspirated and frozen at -20°C. Fresh collagen production medium was added to the flask containing the cells, and they were returned to the incubator maintained at a temperature of 37.0±0.5°C with an atmosphere of air enriched with 10.0±0.5% carbon dioxide. Collagen production medium was changed every 3-4 days and the collagens were harvested from the pericellular layer by extracting in Dulbecco's phosphate buffered saline as described above at 7, 21, 28, 35, and 42 days after changing to production medium. Each time after harvesting the collagens with Dulbecco's phosphate buffered saline, freshly prepared collagen production medium was added to the culture. After the fifth harvest at 42 days in production medium the culture was terminated and the cells were scraped into aqueous acetic acid (5 ml, 0.5M; J.T. Baker Inc., Phillipsburg, NJ) using a cell scraper (Costar Corporation, Cambridge, MA). The acetic acid, containing the scraped cells, was placed in a centrifuge tube (25x89mm ultratube, open-top, thick wall polyallomer centrifuge tube, Nalge Company, Rochester, NY), to solubilize the acetic acid soluble collagens in the cell layer. The scraped cell layer was left in the acetic acid at 4-8°C overnight and then centrifuged in a Beckman high-speed centrifuge (Model J2-21, Beckman Instruments Inc., Palo Alto, CA) at 31,000g for one hour. The supernatant from each sample was aspirated from the pellet and frozen at -20°C. The pellet, containing acetic acid insoluble collagens, cell debris and other insoluble material was also frozen at -20°C.

To examine the collagen production in these cultures the Dulbecco's phosphate buffered saline containing the solubilized collagens and the acetic acid supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to identify the presence of collagen alpha-chains. Aliqouts of the Dulbecco's phosphate buffered saline containing the solubilized collagens from each cold cycle, the acetic acid soluble material from the cell layer, which was neutralized with sodium hydroxide, and a human collagen

I standard, pepsin extracted from human placenta (Sigma type VIII collagen, Sigma Chemical Company, St. Louis, MO) were taken and denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v; Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, w/v) buffer system at 125V (constant voltage) for 2.25 hours. Laemmli, *Nature*, 227:680-685 (1970). The gel was stained with Page Blue 83 (0.1% w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel, shown in figure 8, revealed that human collagen was extracted from the cell layer with Dulbecco's phosphate-buffered saline at 4-8°C, as judged by the presence of bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gel. Also, collagen alpha-chains were observed in the acetic acid soluble material obtained from the cell layer. No other bands were revealed by Page Blue 83 staining on the gel at the loadings used. These results indicate that the cell strain HDF B116, a human neonatal foreskin fibroblast, was capable of producing human collagen by this method.

Example 15: Production of collagen by human fibroblasts, cell strain HDF B117, cultured in serum-free medium in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of human collagen from the cell layer by Dulbecco's phosphate-buffered saline.

Human neonatal foreskin fibroblasts, (designated HDF B117, originated at Organogenesis Inc. Canton, MA), were seeded at $5x10^5$cells/75cm^2 in four plastic tissue culture-grade flask (T75, Costar Company, Cambridge, MA) in serum-free, antibiotic-free growth medium, as described below. The serum-free growth medium contained: Dulbecco's modified Eagle's medium (high glucose

formulation, without L-glutamine; BioWhittaker, Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [4x10 $^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3', 5-triiodo-L-thyronine [2x10^{-11}M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [1x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), *ortho-* phosphorylethanolamine [1x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), adenine [1.8x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), selenious acid [5.3x10^{-8}M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY). The cells were maintained in this growth medium in an incubator at a temperature of 37.0±0.5°C with an atmosphere of 10.0±0.5% carbon dioxide in air. The growth medium was replaced every 3-4 days with freshly prepared growth medium. After eighteen days the cells had become highly confluent, that is the cells had formed a densely packed layer at the bottom of the tissue culture flask. At this time the growth medium was removed and replaced with collagen production medium, which contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [4x10^{-3}M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 µg/ml] (Sigma Chemical Co., St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), 3,3',5-triiodo-L-thyronine [2x10^{-11}M] (Sigma Chemical Co., St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Co., St. Louis, MO), ethanolamine [1x10^{-4}M] (Sigma Chemical Co., St. Louis, MO), *ortho*-phosphorylethanolamine [1x10^{-4}M] (Sigma Chemical Co., St. Louis, MO), selenious acid [5.3x10^{-8}M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI), L-ascorbic acid

phosphate magnesium salt n-hydrate [50µg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}$M, final concentration] (Sigma Chemical Co., St. Louis, MO) and glycine [$1.67x10^{-3}$M, final concentration] (Sigma Chemical Co., St. Louis, MO). The collagen production medium was replaced every 3-4 days with freshly prepared collagen production medium. After seven days, cold cycling to harvest the collagen in Dulbecco's phosphate-buffered saline was performed. Spent collagen production medium was aspirated and cells were rinsed for one minute with 5ml of room temperature Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) to remove any residual medium. The Dulbecco's phosphate buffered saline was aspirated thereafter. To solubilize the pericellular collagens, 5ml of 4-8°C Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) was added to each flask and the flask was placed at 4-8°C for one hour. After one hour, the Dulbecco's phosphate buffered saline, containing collagens solubilized from the cell layer, was aspirated and frozen at -20°C. Fresh collagen production medium was added to the flask containing the cells, and they were returned to the incubator at 37.0±0.5°C and 10.0±0.5% carbon dioxide in air. Collagen production medium was changed every 3-4 days and the collagens were harvested from the pericellular layer by extracting in Dulbecco's phosphate buffered saline as described above weekly. Each time after harvesting the collagens with Dulbecco's phosphate buffered saline, collagen production medium was added to the culture. After the fifth harvest, the cells were scraped into aqueous acetic acid (5 ml, 0.5M; J.T. Baker Inc., Phillipsburg, NJ) using a cell scraper (Costar Corporation, Cambridge, MA). The acetic acid, containing the scraped cells, was placed in a centrifuge tube (16x76mm ultratube, open-top thick wall, polycarbonate, Nalge Company, Rochester, NY), to solubilize the acetic acid soluble collagens in the cell layer. The scraped cell layer was left in the acetic acid at 4-8°C overnight and then centrifuged in a Beckman ultracentrifuge (Model L8-70M, Beckman Instruments Inc., Palo Alto, CA) at 20,000g for 30 minutes.

The supernatant from each sample was aspirated from the pellet and frozen at -20°C. The pellet, containing acetic acid insoluble collagens, cell debris and other insoluble material was also frozen at -20°C.

To examine the collagen production by these cultures samples of the Dulbecco's phosphate buffered saline containing the solubilized collagens and the acetic acid supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to identify the presence of collagen alpha-chains. Aliqouts of the Dulbecco's phosphate buffered saline containing the solubilized collagens from each cold cycle and the acetic acid extraction of the cell layer were taken and denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, v/v) buffer system at 125V (constant voltage) for 2.25 hours. Laemmli, *Nature*, 227:680-685 (1970). The gel was stained with Page Blue 83 (0.1% w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel, shown in figure 9, revealed that human collagen was extracted from the cell layer with Dulbecco's phosphate-buffered saline at 4-8°C, as judged by the presence of bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gel. Also, collagen alpha-chains were observed in the acetic acid soluble material obtained from the cell layer. No other bands were revealed by Page Blue 83 staining on the gel at the loadings used. These results indicate that the cell strain HDF B117, a human neonatal foreskin fibroblast, was capable of producing human collagen by this method.

Example 16: Production of collagen by sheep arterial fibroblasts, cell strain SAF 012A, cultured in serum-free medium in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of

collagen from the cell layer by Dulbecco's phosphate-buffered saline.

Sheep arterial fibroblasts (designated SAF 012A, originated at Organogenesis Inc., Canton, MA) were seeded at $5x10^5$cells/75cm^2 in four plastic tissue culture-grade flask (T75, Costar Company, Cambridge, MA) in serum-free, antibiotic-free growth medium, as described below. The serum-free growth medium contained: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker, Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3', 5-triiodo-L-thyronine [$2x10^{-11}$M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), adenine [$1.8x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}$M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY). The cells were maintained in this growth medium in an incubator at a temperature of 37.0±0.5°C with an atmosphere of 10.0±0.5% carbon dioxide in air. The growth medium was replaced every 3-4 days with freshly prepared growth medium. After ten days the cells had become highly confluent, that is the cells had formed a densely packed layer at the bottom of the tissue culture flask. At this time the growth medium was removed and replaced with collagen production medium, which contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL,

Gaithersburg, MD), hydrocortisone [0.4μg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5μg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$] (Sigma Chemical Company, St. Louis, MO), human transferrin [5μg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}M$] (Sigma Chemical Company, St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}M$] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}M$] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50μg/ml] (Aldrich Chemical Co., Milwaukee, WI), L-ascorbic acid phosphate magnesium salt n-hydrate [50μg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}M$, final concentration] (Sigma Chemical Co., St. Louis, MO) and glycine [$1.67x10^{-3}M$, final concentration] (Sigma Chemical Co., St. Louis, MO). The collagen production medium was replaced every 3-4 days with freshly prepared collagen production medium. After seven days, cold cycling to harvest the collagen in Dulbecco's phosphate-buffered saline was performed. Spent collagen production medium was aspirated and cells were rinsed for one minute with 5ml of room temperature Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) to remove any residual medium. The Dulbecco's phosphate buffered saline was aspirated thereafter. To solubilize the pericellular collagens, 5ml of 4-8°C Dulbecco's phosphate buffered saline with calcium and magnesium (Catalog #17-513Q; BioWhittaker Inc., Walkersville, MD) was added to each flask and the flask was placed at 4-8°C for one hour. After one hour, the Dulbecco's phosphate buffered saline, containing collagens solubilized from the cell layer, was aspirated and frozen at -20°C. Fresh collagen production medium was added to the flask containing the cells, and they were returned to the incubator at 37.0±0.5°C with an atmosphere of air enriched with 10.0±0.5% carbon dioxide. Collagen production medium was changed every 3-4 days and the collagens were harvested from the pericellular layer by extracting in Dulbecco's phosphate buffered saline as described above at 7, 12, 21, 28, and 35 days in production medium. Each time after harvesting the collagens with Dulbecco's

phosphate buffered saline, collagen production medium was added to the culture. After the fifth harvest at 35 days in production medium, the culture was terminated by scraping the cells were into aqueous acetic acid (5 ml, 0.5M; J.T. Baker Inc., Phillipsburg, NJ) using a cell scraper (Costar Corporation, Cambridge, MA). The acetic acid, containing the scraped cells, was placed in a centrifuge tube (16x76mm ultratube, open-top, thick- walled, polycarbonate, Nalge Company, Rochester, NY), to solubilize the acetic acid soluble collagens in the cell layer. The scraped cell layer was left in the acetic acid at 4-8°C overnight and then centrifuged in a Beckman ultracentrifuge (Model L8-70M, Beckman Instruments Inc., Palo Alto, CA) at 20,000g for 30 minutes. The supernatant from each sample was aspirated from the pellet and frozen at -20°C. The pellet, containing acetic acid insoluble collagens, cell debris and other insoluble material was also frozen at -20°C.

To examine the collagen production by these cultures the Dulbecco's phosphate buffered saline containing the solubilized collagens and the acetic acid supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to identify the presence of collagen alpha-chains. Aliqouts of the Dulbecco's phosphate buffered saline from cold cycle washes taken after 21, 28 and 35 days in production medium and the acetic acid extraction of the cell layer were denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, v/v) buffer system at 125V (constant voltage) for 2.25 hours. Laemmli, *Nature*, 227:680-685 (1970). The gel was stained with Page Blue 83 (0.1%, w/v; Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel, shown in figure 10, revealed that collagen was extracted from the cell layer with Dulbecco's phosphate-buffered saline at 4-8°C, as judged by the presence of bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gel. Also, collagen alpha-chains were observed in the acetic

acid soluble material obtained from the cell layer. No other bands were revealed by Page Blue 83 staining on the gel at the loadings used. These results indicate that the cell strain SAF 012A, a sheep arterial fibroblast, was capable of producing collagen by this method.

Example 17: Production of collagen by human fibroblasts, cell strain HDF B119, cultured in serum-free medium in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of human collagen from the cell layer by Dulbecco's phosphate buffered saline without calcium or magnesium.

Human neonatal foreskin fibroblasts (designated HDF B119 originated at Organogenesis Inc., Canton, MA) were seeded at $7x10^5$cells/75cm^2 in four plastic tissue culture-grade flask (T75, Costar Company, Cambridge, MA) in serum-free, antibiotic-free growth medium, as described below. The serum-free growth medium contained: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker, Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3', 5-triiodo-L-thyronine [$2x10^{-11}$M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), *ortho-* phosphorylethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), adenine [$1.8x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}$M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY). The cells were maintained in

this growth medium in an incubator at a temperature of 37.0±0.5°C with an atmosphere of 10.0±0.5% carbon dioxide in air. The growth medium was replaced every 3-4 days with freshly prepared growth medium. After thirteen days the cells had become highly confluent, that is the cells had formed a densely packed layer at the bottom of the tissue culture flask. At this time the growth medium was removed and replaced with collagen production medium, which contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}M$] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}M$] (Sigma Chemical Company, St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}M$] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}M$] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Company, Milwaukee, WI), L-ascorbic acid phosphate magnesium salt n-hydrate [50µg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}M$, final concentration] (Sigma Chemical Company, St. Louis, MO) and glycine [$1.67x10^{-3}M$, final concentration] (Sigma Chemical Company, St. Louis, MO). The collagen production medium was replaced every 3-4 days with freshly prepared collagen production medium. After fifteen days, cold cycling to harvest the collagen in Dulbecco's phosphate buffered saline without calcium or magnesium was performed. Spent collagen production medium was aspirated and cells were rinsed for one minute with 5ml of room temperature Dulbecco's phosphate buffered saline without calcium or magnesium (Catalog # 17-512F; BioWhittaker, Walkersville, MD) to remove any residual medium. The Dulbecco's phosphate buffered saline without calcium or magnesium was aspirated thereafter. To solubilize the pericellular collagens, 5ml of 4-8°C Dulbecco's phosphate buffered

saline without calcium or magnesium (Catalog # 17-512F; BioWhittaker, Walkersville, MD) was added to each flask and the flask was placed at 4-8°C for one hour. After one hour, the Dulbecco's phosphate buffered saline without calcium or magnesium, containing collagens solubilized from the cell layer, was aspirated and frozen at -20°C. Fresh collagen production medium was added to the flask containing the cells, and they were returned to the incubator at 37.0±0.5°C with an atmosphere of air enriched with 10.0±0.5% carbon dioxide. Collagen production medium was changed every 3-4 days and the collagens were harvested from the pericellular layer by extracting in Dulbecco's phosphate buffered saline without calcium or magnesium as described above weekly for five weeks. Each time after harvesting the collagens with Dulbecco's phosphate buffered saline without calcium or magnesium, collagen production medium was added to the culture. After the fifth harvest, the cells were scraped into aqueous acetic acid (5 ml, 0.5M; J.T. Baker Inc., Phillipsburg, NJ) using a cell scraper (Costar Corporation, Cambridge, MA). The acetic acid, containing the scraped cells, was placed in a centrifuge tube (16x76mm ultratube, open-top, thick-walled, polycarbonate, Nalgene Brand, Rochester, NY), to solubilize the acetic acid soluble collagens in the cell layer. The scraped cell layer was left in the acetic acid at 4-8°C overnight and then centrifuged in a Beckman high-speed centrifuge (Model J2-21, Beckman Instruments Inc., Palo Alto, CA) using a Type 40 rotor at 20,000g for 30 minutes. The supernatant from each sample was aspirated from the pellet and frozen at -20°C. The pellet, containing acetic acid insoluble collagens, cell debris and other insoluble material was also frozen at -20°C.

To examine the collagen production by these cultures the Dulbecco's phosphate buffered saline without calcium or magnesium containing the solubilized collagens and the acetic acid supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to identify the presence of collagen alpha-chains. Aliqouts of the Dulbecco's phosphate buffered saline without calcium or magnesium from each cold cycle and the acetic acid extraction of the cell layer, which was neutralized with sodium

hydroxide, were taken and denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, v/v) buffer system at 125V (constant voltage) for 2.25 hours. Laemmli, *Nature*, 227:680-685 (1970). The gel was stained with Page Blue 83 (0.1% w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel revealed that human collagen was extracted from the cell layer with Dulbecco's phosphate-buffered saline at 4-8°C, as judged by the presence of bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gel. Also, collagen alpha-chains were observed in the acetic acid soluble material obtained from the cell layer. No other bands were revealed by Page Blue 83 staining on the gel at the loadings used. These results indicate that the collagen deposited in the cell layer in the presence of beta-aminopropionitrile was able to be solubilized with Dulbecco's phosphate buffered saline without calcium or magnesium.

Example 18: Production of collagen by human fibroblasts, cell strain HDF B119, cultured in serum-free medium in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of human collagen from the cell layer by phosphate buffered saline without calcium or magnesium.

Human neonatal foreskin fibroblasts, (designated HDF B119, originated at Organogenesis Inc., Canton, MA) were seeded at $7x10^5$cells/$75cm^2$ in four plastic tissue culture-grade flask (T75, Costar Company, Cambridge, MA) in serum-free, antibiotic-free growth medium, as described below. The serum-free growth medium contained: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker, Walkersville, MD)

and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [4x10 $^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4μg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5μg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3', 5-triiodo-L-thyronine [2x10^{-11}M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5μg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [1x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), *ortho*- phosphorylethanolamine [1x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), adenine [1.8x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), selenious acid [5.3x10^{-8}M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50μg/ml] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY). The cells were maintained in this growth medium in an incubator at a temperature of 37.0±0.5°C with an atmosphere of 10.0±0.5% carbon dioxide in air. The growth medium was replaced every 3-4 days with freshly prepared growth medium. After thirteen days the cells had become highly confluent, that is the cells had formed a densely packed layer at the bottom of the tissue culture flask. At this time the growth medium was removed and replaced with collagen production medium, which contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [4x10^{-3}M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 μg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5μg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3',5-triiodo-L-thyronine [2x10^{-11}M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5μg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [1x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), *ortho*-phosphorylethanolamine [1x10^{-4}M] (Sigma Chemical Company, St. Louis, MO), selenious acid [5.3x10^{-8}M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50μg/ml] (Aldrich Chemical Company, Milwaukee, WI), L-ascorbic acid

phosphate magnesium salt n-hydrate [50µg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}$M, final concentration] (Sigma Chemical Company, St. Louis, MO) and glycine [$1.67x10^{-3}$M, final concentration] (Sigma Chemical Company, St. Louis, MO). The collagen production medium was replaced every 3-4 days with freshly prepared collagen production medium. After fifteen days, cold cycling to harvest the collagen in phosphate buffered saline without calcium or magnesium was performed. Spent collagen production medium was aspirated and cells were rinsed for one minute with 5ml of room temperature phosphate buffered saline without calcium or magnesium (Catalog # 17-516B; BioWhittaker, Walkersville, MD) to remove any residual medium. The phosphate buffered saline without calcium or magnesium was aspirated thereafter. To solubilize the pericellular collagens, 5ml of 4-8°C phosphate buffered saline without calcium or magnesium (Catalog # 17-516B; BioWhittaker, Walkersville, MD) was added to each flask and the flask was placed at 4-8°C for one hour. After one hour, the phosphate buffered saline without calcium or magnesium, containing collagens solubilized from the cell layer, was aspirated and frozen at -20°C. Fresh collagen production medium was added to the flask containing the cells, and they were returned to the incubator at 37.0±0.5°C with an atmosphere of air enriched with 10.0±0.5% carbon dioxide. Collagen production medium was changed every 3-4 days and the collagens were harvested from the pericellular layer by extracting in phosphate buffered saline without calcium or magnesium as described above weekly for five weeks. Each time after harvesting the collagens with phosphate buffered saline without calcium or magnesium, collagen production medium was added to the culture. After the fifth harvest, the cells were scraped into aqueous acetic acid (5 ml, 0.5M; J.T. Baker Inc., Phillipsburg, NJ) using a cell scraper (Costar Corporation, Cambridge, MA). The acetic acid, containing the scraped cells, was placed in a centrifuge tube (16x76mm ultratube, open-top, thick-walled, polycarbonate, Nalgene Brand, Rochester, NY), to solubilize the acetic acid soluble collagens in the cell layer. The scraped cell layer was left in the acetic acid at 4-8°C overnight and then

centrifuged in a Beckman high-speed centrifuge (Model J2-21, Beckman Instruments Inc., Palo Alto, CA) using a Type 40 rotor at 20,000g for 30 minutes. The supernatant from each sample was aspirated from the pellet and frozen at -20°C. The pellet, containing acetic acid insoluble collagens, cell debris and other insoluble material was also frozen at -20°C.

To examine the collagen production by these cultures the phosphate buffered saline without calcium or magnesium containing the solubilized collagens and the acetic acid supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to identify the presence of collagen alpha-chains. Aliqouts of the phosphate buffered saline without calcium or magnesium from each cold cycle and the acetic acid extraction of the cell layer, which was neutralized with sodium hydroxide, were taken and denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, v/v) buffer system at 125V (constant voltage) for 2.25 hours. Laemmli, *Nature*, 227:680-685 (1970). The gel was stained with Page Blue 83 (0.1% w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel revealed that human collagen was extracted from the cell layer with phosphate buffered saline without calcium and magnesium at 4-8°C, as judged by the presence of bands that migrated as collagen alpha1(I)- and alpha2(I)-chains on the gel. Also, collagen alpha-chains were observed in the acetic acid soluble material obtained from the cell layer. No other bands were revealed by Page Blue 83 staining on the gel at the loadings used. These results indicate that the collagen deposited in the cell layer in the presence of beta-aminopropionitrile was able to be solubilized with phosphate buffered saline without calcium or magnesium.

Example 19: Production of collagen by human fibroblasts, cell strain HDF B119, cultured in serum-free medium in the presence of a lysyl oxidase inhibitor, beta-aminopropionitrile, subjected to routine extractions of human collagen from the cell layer by a 0.3M solution of sodium chloride.

Human neonatal foreskin fibroblasts (designated HDF B119, originated at Organogenesis Inc., Canton, MA) were seeded at $7x10^5$cells/75cm^2 in four plastic tissue culture-grade flask (T75, Costar Company, Cambridge, MA) in serum-free, antibiotic-free growth medium, as described below. The serum-free growth medium contained: Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker, Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}$M] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3', 5-triiodo-L-thyronine [$2x10^{-11}$M] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), *ortho-* phosphorylethanolamine [$1x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), adenine [$1.8x10^{-4}$M] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}$M] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Co., Milwaukee, WI) and human recombinant epidermal growth factor [5ng/ml] (Upstate Biotechnology Inc., Lake Placid, NY). The cells were maintained in this growth medium in an incubator at a temperature of 37.0±0.5°C with an atmosphere of 10.0±0.5% carbon dioxide in air. The growth medium was replaced every 3-4 days with freshly prepared growth medium. After thirteen days the cells had become highly confluent, that is the cells had formed a densely packed layer at the bottom of the tissue culture flask. At this time the growth medium was removed and replaced with collagen production medium, which

contained Dulbecco's modified Eagle's medium (high glucose formulation, without L-glutamine; BioWhittaker Inc., Walkersville, MD) and Nutrient Mix F-12 Ham's (without L-glutamine; Hyclone Labs, Logan, UT) mixed in a 3:1 (v/v) ratio supplemented with GlutaMAX-I [$4x10^{-3}M$] (Gibco BRL, Gaithersburg, MD), hydrocortisone [0.4 µg/ml] (Sigma Chemical Company, St. Louis, MO), bovine insulin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), 3,3',5-triiodo-L-thyronine [$2x10^{-11}M$] (Sigma Chemical Company, St. Louis, MO), human transferrin [5µg/ml] (Sigma Chemical Company, St. Louis, MO), ethanolamine [$1x10^{-4}M$] (Sigma Chemical Company, St. Louis, MO), *ortho*-phosphorylethanolamine [$1x10^{-4}M$] (Sigma Chemical Company, St. Louis, MO), selenious acid [$5.3x10^{-8}M$] (Aldrich Chemical Company, Milwaukee, WI), beta-aminopropionitrile [50µg/ml] (Aldrich Chemical Company, Milwaukee, WI), L-ascorbic acid phosphate magnesium salt n-hydrate [50µg/ml] (Wako Pure Chemical Industries, Ltd., Richmond, VA), L-proline [$1.93x10^{-3}M$, final concentration] (Sigma Chemical Company, St. Louis, MO) and glycine [$1.67x10^{-3}M$, final concentration] (Sigma Chemical Company, St. Louis, MO). The collagen production medium was replaced every 3-4 days with freshly prepared collagen production medium. After fifteen days, cold cycling to harvest the collagen in a solution of sodium chloride was performed. Spent collagen production medium was aspirated and cells were rinsed for one minute with 5ml of room temperature sodium chloride solution (0.3M; Mallinckrodt Specialty Chemicals, Chesterfield, MO) to remove any residual medium. The solution of sodium chloride was aspirated thereafter. To solubilize the pericellular collagens, 5ml of a 4-8°C solution of sodium chloride (0.3M; Mallinckrodt Specialty Chemicals, Chesterfield, MO) was added to each flask and the flask was placed at 4-8°C for one hour. After one hour, the solution of sodium chloride containing collagens solubilized from the cell layer, was aspirated and frozen at -20°C. Fresh collagen production medium was added to the flask containing the cells, and they were returned to the incubator at 37.0±0.5°C with an atmosphere of air enriched with 10.0±0.5% carbon dioxide. Collagen production medium was changed every 3-4 days and the collagens were harvested from the

pericellular layer by extracting in a solution of sodium chloride as described above weekly for five weeks. Each time after harvesting the collagens with a solution of sodium chloride, collagen production medium was added to the culture. After the fifth harvest, the cells were scraped into aqueous acetic acid (5 ml, 0.5M; J.T. Baker Inc., Phillipsburg, NJ) using a cell scraper (Costar Corporation, Cambridge, MA). The acetic acid, containing the scraped cells, was placed in a centrifuge tube (16x76mm ultratube, open-top, thick-walled, polycarbonate, Nalgene Brand, Rochester, NY), to solubilize the acetic acid soluble collagens in the cell layer. The scraped cell layer was left in the acetic acid at 4-8°C overnight and then centrifuged in a Beckman high-speed centrifuge (Model J2-21, Beckman Instruments Inc., Palo Alto, CA) using a Type 40 rotor at 20,000g for 30 minutes. The supernatant from each sample was aspirated from the pellet and frozen at -20°C. The pellet, containing acetic acid insoluble collagens, cell debris and other insoluble material was also frozen at -20°C.

To examine the collagen production by these cultures the solution of sodium chloride containing the solubilized collagens and the acetic acid supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to identify the presence of collagen alpha-chains. Aliqouts of the solution of sodium chloride from each cold cycle and the acetic acid extraction of the cell layer, which was neutralized with sodium hydroxide, were taken and denatured in sodium dodecyl sulfate (17.4%, w/v), glycerol (6%, w/v) and beta-mercaptoethanol (1.5%, v/v) at 100°C for three minutes and then separated by electrophoresis in a polyacrylamide minigel (8%, w/v, Novex, San Diego, CA) using a Tris (0.025M)/glycine (0.192M)/sodium dodecyl sulfate (0.1%, v/v) buffer system at 125V (constant voltage) for 2.25 hours. Laemmli, *Nature*, 227:680-685 (1970). The gel was stained with Page Blue 83 (0.1% w/v, Fluka, Ronkonkoma, NY) in methanol/acetic acid/water (5:1:4) for 1-2 hours. Diffusional destaining was with aqueous acetic acid (8%, v/v) for 24-48 hours. The gel revealed that human collagen was extracted from the cell layer with a solution of sodium chloride at 4-8°C, as judged by the presence of bands that migrated as collagen

alpha1(I)- and alpha2(I)-chains on the gel. Also, collagen alpha-chains were observed in the acetic acid soluble material obtained from the cell layer. No other bands were revealed by Page Blue 83 staining on the gel at the loadings used. These results indicate that the collagen deposited in the cell layer in the presence of beta-aminopropionitrile was able to be solubilized with a solution of sodium chloride (0.3M).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What Is Claimed Is:

1. A method of producing collagen from a collagen-producing cell comprising:

(a) incubating a collagen-producing cell in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions to grow said cells such that collagen is synthesized from said cells;

(b) solubilizing said collagen under conditions that maintain viable cells;

(c) removing said solubilized collagen; and

(d) continued incubation of said cells in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions sufficient to grow said cells such that collagen is synthesized from said cells;

(e) solubilizing said collagen under conditions that maintain viable cells; and

(f) removing said solubilized collagen.

2. The method of claim 1 wherein said cells are selected from the group consisting of fibroblasts, smooth muscle cells, endothelial and epithelial cells.

3. The method of claim 1 wherein said cells are fibroblast cells.

4. The method of claim 1 wherein said collagen-producing cells are derived from human sources.

5. The method of claim 1 wherein said collagen-producing cells are derived from mammalian sources.

6. The method of claim 1 wherein said collagen-producing cells are derived from avian sources.

7. The method of claim 1 wherein said collagen-producing cells are derived from fish sources.

8. The method of claim 1 wherein said collagen-producing cells are derived from invertebrate sources.

9. The method of claim 1 wherein said cells are spontaneously or virally transformed cells.

10. The method of claim 1 wherein said collagen-producing cells are genetically engineered.

11. The method of claim 1 wherein the medium is chemically defined.

12. The method of claim 1 wherein said agent to inhibit or to interfere with collagen crosslinking is selected from the group consisting of β-aminopropionitrile, 2-bromoethylamine hydrobromide, *cis*-1,2-diaminocyclohexane, *trans*-2-phenylcyclopropylamine hydrochloride (tranylcypromine), 2-nitroethylamine hydrochloride, and 2-chloroethylamine hydrochloride.

13. The method of claim 1 wherein said agent to inhibit or to interfere with collagen crosslinking is D-penicillamine.

14. A method of producing collagen from a collagen-producing cell comprising:

(a) incubating a collagen-producing cell in a culture medium containing an agent to inhibit or to interfere with collagen crosslinking under conditions to grow said cells such that collagen is synthesized from said cells;

(b) removing said culture medium from said cells and washing said cells with a saline solution to dissolve said collagen under conditions that maintain viable cells;

(c) removing said saline solution; and

(d) continued incubation of said cells in a culture medium containing an agent to inhibit or to interfere with collagen crosslinking under conditions sufficient to grow said cells such that collagen is synthesized from said cells; and

(e) continued removal of said culture medium from said cells and continued washing of said cells with a saline solution to dissolve said collagen under conditions that maintain viable cells; and

(f) continued recovery of said collagen from said saline solution.

15. The method of claim 14 wherein said cells are selected from the group consisting of fibroblasts, smooth muscle cells, endothelial and epithelial cells.

16. The method of claim 14 wherein said cells are fibroblast cells.

17. The method of claim 14 wherein said collagen-producing cells are derived from human sources.

18. The method of claim 14 wherein said collagen-producing cells are derived from mammalian sources.

19. The method of claim 14 wherein said collagen-producing cells are derived from avian sources.

20. The method of claim 14 wherein said collagen-producing cells are derived from fish sources.

21. The method of claim 14 wherein said collagen-producing cells are derived from invertebrate sources.

22. The method of claim 14 wherein said cells are spontaneously or virally transformed cells.

23. The method of claim 14 wherein said collagen-producing cells are genetically engineered.

24. The method of claim 14 wherein the medium is chemically defined.

25. The method of claim 14 wherein said agent to inhibit or to interfere with collagen crosslinking is selected from the group consisting of ß-aminopropionitrile, 2-bromoethylamine hydrobromide, cis-1,2-diaminocyclohexane, trans-2-phenylcyclopropylamine hydrochloride (tranylcypromine), 2-nitroethylamine hydrochloride, and 2-chloroethylamine hydrochloride.

26. The method of claim 14 wherein said agent to inhibit or to interfere with collagen crosslinking is D-penicillamine.

27. The method of claim 14 wherein said saline solution is selected from the group consisting of Dulbecco's phosphate-buffered saline with calcium and magnesium, Dulbecco's phosphate-buffered saline without calcium and magnesium, 0.3 M sodium chloride, Earle's balanced salt solution and Hank's balanced salt solution.

28. A non-crosslinked, salt soluble collagen that contains no propeptides (the N- and C- propeptides), contains telopeptides, has greater than 40% of the translated proline residues within the

triple-helical region of the molecule hydroxylated, and contains two alpha1(I)-chains and one alpha2(I)-chain.

29. A non-crosslinked, salt soluble collagen that contains no propeptides (the N- and C- propeptides), has no telopeptides, has greater than 40% of the translated proline residues within the triple-helical region of the molecule hydroxylated, and contains two alpha1(I)-chains and one alpha2(I)-chain.

30. The collagen of claims 1 or 14 wherein said collagen is human collagen.

31. A noncrosslinked salt soluble human collagen I made by the method comprising:

(a) incubating a collagen-producing cell in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions to grow said cells such that collagen is synthesized from said cells;

(b) solubilizing said collagen under conditions that maintain viable cells;

(c) removing said solubilized collagen; and

(d) continued incubation of said cells in a culture medium containing an agent to inhibit or to interfere with collagen crosslinking under conditions sufficient to grow said cells such that collagen is synthesized from said cells;

(e) solubilizing said collagen under conditions that maintain viable cells; and

(f) removing said solubilized collagen,

wherein said collagen contains no propeptides (the N- and C-propeptides), contains telopeptides, has greater than 40% of the translated proline residues within the triple-helical region of the molecule hydroxylated, and contains two alpha1(I)-chains and one alpha2(I)-chain.

32. A noncrosslinked salt soluble human collagen I made by the method comprising:

(a) incubating a collagen-producing cell in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions to grow said cells such that collagen is synthesized from said cells;

(b) removing said culture medium from said cells and washing said cells with a dilute saline solution to dissolve said collagen;

(c) removing said dilute saline solution; and

(d) continued incubation of said cells in a culture medium containing a lysyl oxidase inhibitor under conditions sufficient to grow said cells such that collagen is synthesized from said cells; and

(e) continued removal of said culture medium from said cells and continued washing of said cells with a dilute saline solution to dissolve said collagen; and

(f) removing said dilute saline solution,

wherein said collagen contains no propeptides (the N- and C-propeptides), contains telopeptides, has greater than 40% of the translated proline residues within the triple-helical region of the molecule hydroxylated, and contains two alpha1(I)-chains and one alpha2(I)-chain.

33. Procollagen made by the method comprising:

(a) incubating a collagen-producing cell in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions to grow said cells such that collagen is synthesized from said cells;

(b) removing said cell culture medium containing procollagen from said cell culture;

(c) solubilizing said collagen under conditions that maintain viable cells;

(d) removing said solubilized collagen; and

(e) continued incubation of said cells in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions sufficient to grow said cells such that collagen is synthesized from said cells;

(f) removing said cell culture medium containing procollagen from said cell culture;

(g) solubilizing said collagen under conditions that maintain viable cells.

34. The method of claim 33 wherein said cells are selected from the group consisting of fibroblasts, smooth muscle cells, endothelial and epithelial cells.

35. The method of claim 33 wherein said cells are fibroblast cells.

36. The method of claim 33 wherein said collagen-producing cells are derived from human sources.

37. The method of claim 33 wherein said collagen-producing cells are derived from mammalian sources.

38. The method of claim 33 wherein said collagen-producing cells are derived from avian sources.

39. The method of claim 33 wherein said collagen-producing cells are derived from fish sources.

40. The method of claim 33 wherein said collagen-producing cells are derived from invertebrate sources.

41. The method of claim 33 wherein said cells are spontaneously or virally transformed cells.

42. The method of claim 33 wherein said collagen-producing cells are genetically engineered.

43. The method of claim 33 wherein the medium is chemically defined.

44. The collagen propeptides of claim 33 wherein said collagen propeptides are human collagen propeptides.

45. The method of claim 1 or 14, further comprising:

(g) recovering non-salt soluble collagen from the cultured cells.

46. The method of claim 45 wherein said non-salt soluble collagen is solubilized by extracting said culture into dilute acetic acid and collected by differential salt precipitation.

47. The method of producing collagen from a collagen-producing cell comprising:

(a) incubating a collagen-producing cell in a culture medium under conditions sufficient to grow said cells such that collagen is synthesized from said cells;

(b) solubilizing said collagen under conditions that maintain viable cells; and

(c) removing said solubilized collagen,

wherein said collagen-producing cell is genetically engineered such that said cell's ability to produce lysyl oxidase is prevented or reduced.

48. The method of claim 47 wherein the lysyl oxidase gene of said cell has been deleted or altered such that said lysyl oxidase cannot be produced.

49. The method of claim 47 wherein said cell contains an antisense construct specific for the lysyl oxidase gene such that said lysyl oxidase cannot be produced.

50. The method of claim 47 wherein the lysine residues in the telopeptides of the produced collagen have been altered or changed such that lysyl oxidase will not recognize the telopeptide region as a substrate.

51. A method of producing human collagen III from a collagen-producing cell comprising:

(a) incubating a collagen-producing cell in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions to grow said cells such that collagen is synthesized from said cells;

(b) solubilizing said collagen under conditions that maintain viable cells;

(c) removing said solubilized collagen; and

(d) continued incubation of said cells in a culture medium containing an agent to inhibit or interfere with collagen crosslinking under conditions sufficient to grow said cells such that collagen is synthesized from said cells;

(e) solubilizing said collagen under conditions that maintain viable cells; and

(f) removing said solubilized human collagen III.

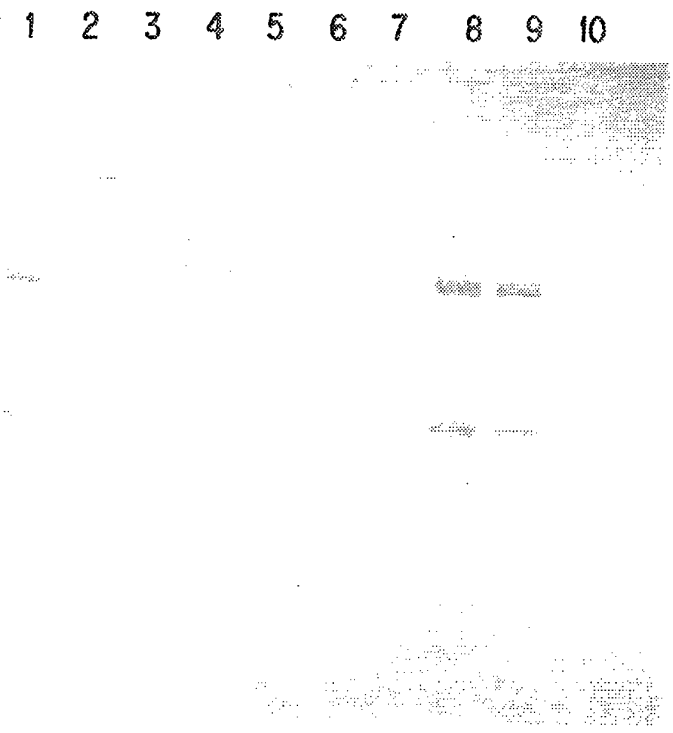

FIG. 1

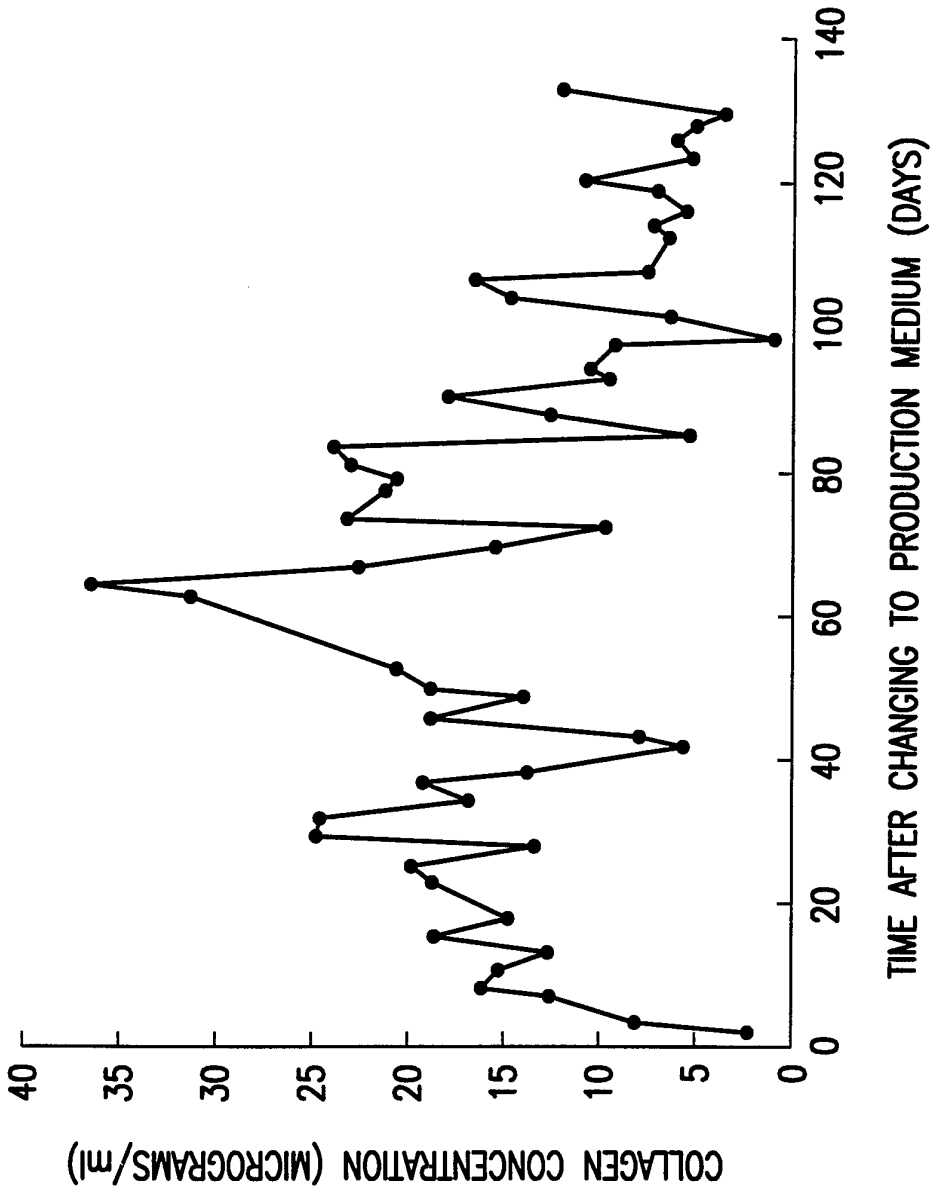
COLLAGEN CONCENTRATION (MICROGRAMS/ml)
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
120
140
TIME AFTER CHANGING TO PRODUCTION MEDIUM (DAYS)

FIG.2

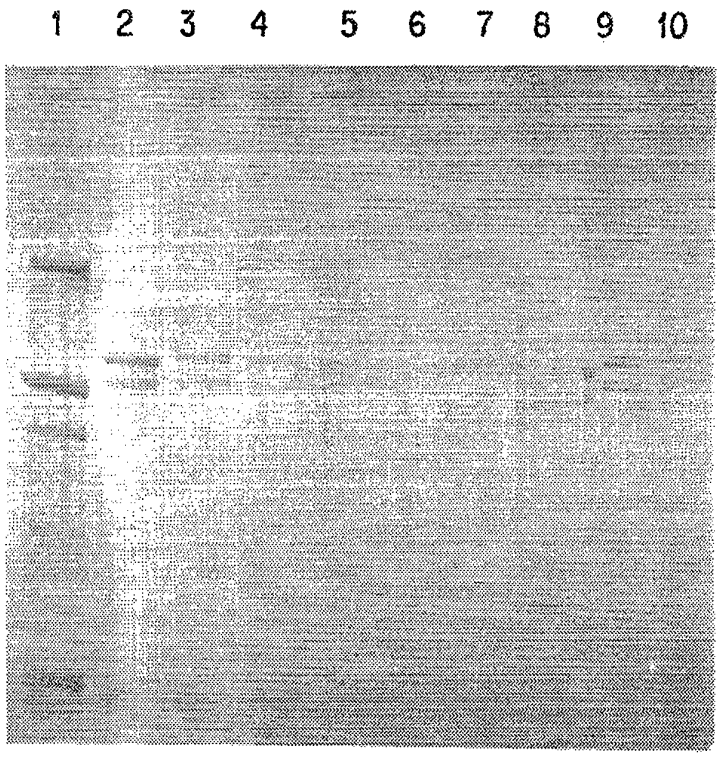

FIG. 3



FIG. 4



FIG.5

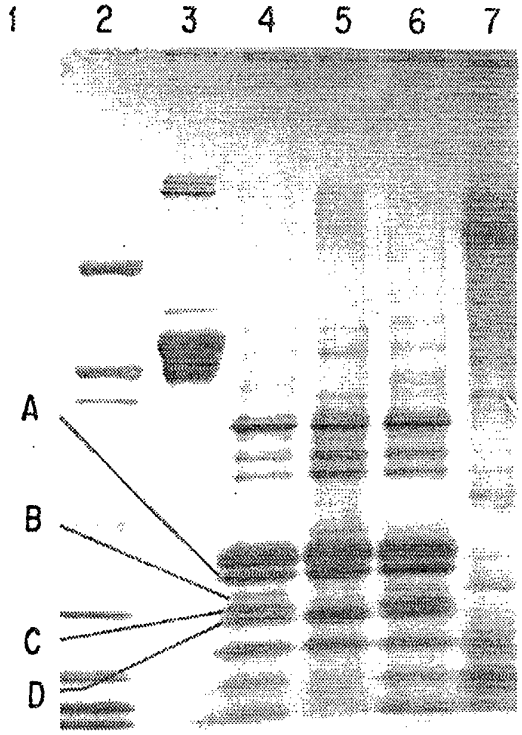

FIG.6

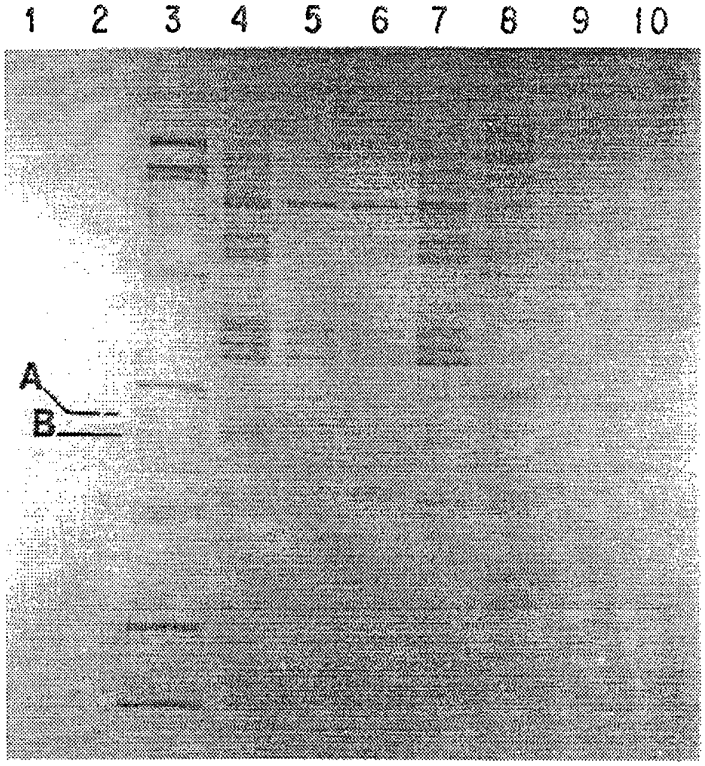

FIG. 7

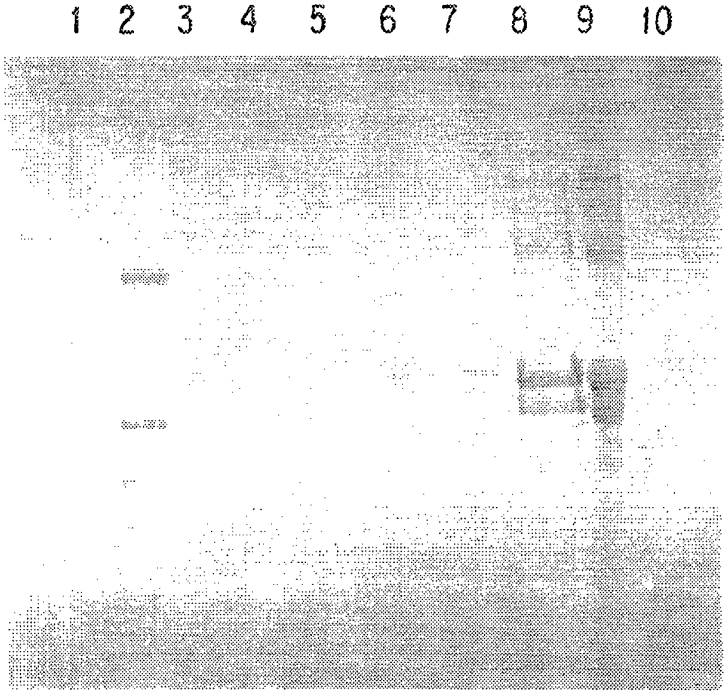

FIG. 8

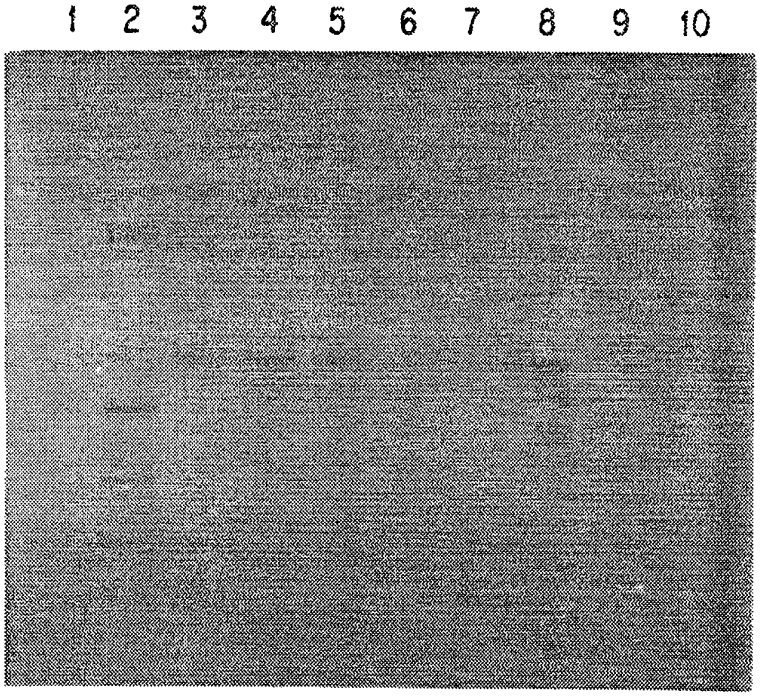

FIG. 9

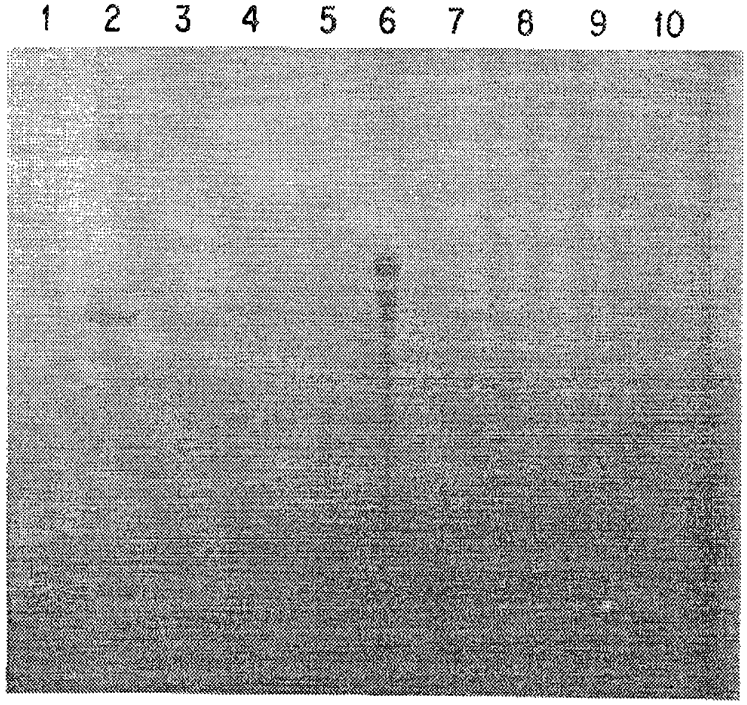

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05855

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/14, 14/78; C12N 5/06, 5/08, 5/10
US CL :435/69.1, 70.1, 70.3, 240.2, 273, 804, 813; 530/356, 412
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 70.1, 70.3, 240.2, 273, 804, 813; 530/356, 412

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

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

APS, BIOSIS, CA, INPADOC, JICST-E search terms: collagen, non-crosslink, lathyritic, cell culture, extraction

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EXPERIMENTAL CELL RESEARCH, Volume 181, issued 1989, Grinnell et al, "Collagen Processing, Crosslinking, and Fibril Bundle Assembly in Matrix Produced by Fibroblasts in Long-Term Cultures Supplemented with Ascorbic Acid", pages 483-491, see page 486, paragraph 4.	1-8,11-21,24-40,45,46,51
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, Number 4, issued 05 February 1992, Torre-Blanco et al, "Temperature-induced Post-translational Over-modification of Type I Procollagen", pages 2650-2655, see pages 2651-2652.	1-8,11-21,24-40,45,46,51

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 JULY 1995	Date of mailing of the international search report 23AUG1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN WALSH Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05855

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ACTA BIOCHIMICA POLONICA, Volume 27, Number 3/4, issued 1980, Bankowski et al, "Stimulation Of Collagen Biosynthesis By Platelet Homogenate In Various Cell Cultures", pages 405-411, see page 406, paragraph 3.	1-8,11-21, 24-40,45,46, 51
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 266, Number 22, issued 05 August 1991, Ala-Kokko et al, "Expression of a Human Cartilage Procollagen Gene (COL2A1) in Mouse 3T3 Cells", pages 14175-14178, see Materials and Methods section bridging pages 14175-14176.	9,10,22,23,41-44
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 266, Number 2, issued 15 January 1991, Olsen et al, "High Levels of Expression of a Minigene Version of the Human Proα1(I) Collagen Gene in Stably Transfected Mouse Fibroblasts", pages 1117-1121, see Materials and Methods section at page 1118.	9,10,22,23,41-44
Y	METHODS IN ENZYMOLOGY, Volume 82, issued 1982, Miller et al, "Preparation and Characterization of the Different Types of Collagen", pages 33-64, see entire document.	1-46,51
Y,P	US, A, 5,405,757 (PROCKOP ET AL) 11 April 1995, see entire document.	1-46,51
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 244, Number 7, issued 10 April 1969, Deshmukh et al, "A Defect in the Intramolecular and Intermolecular Cross-linking of Collagen Caused by Penicillamine", pages 1787-1795, see page 1787, first paragraph.	1-51