



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/00, C07K 5/00, 7/00, 16/00, 17/00, G01N 33/53, 33/537, 33/542, 33/543, 33/567	A1	(11) International Publication Number: WO 98/56399 (43) International Publication Date: 17 December 1998 (17.12.98)
(21) International Application Number: PCT/US98/12327 (22) International Filing Date: 12 June 1998 (12.06.98) (30) Priority Data: 60/049,369 12 June 1997 (12.06.97) US (71) Applicants: FIBROGEN, INC. [US/US]; 225 Gateway Boulevard, South San Francisco, CA 94080 (US). ACADEMY OF FINLAND [FI/FI]; Vilhonvuorenkatu 6, FIN-00550 Helsinki (FI). INSTITUT DE LA SANTE ET DE LA RECHERCHE MEDICALE [FR/FR]; 101, rue de Tolbiac, F-75654 Paris Cedex 13 (FR). (72) Inventors: PIHLAJANIEMI, Taina; Nauriskuja Runkotie 1B3, FIN-90460 Oulunsalo (FI). REHN, Marko; Apajatie 2A4, FIN-90220 Oulo (FI). CLEMENT, Bruno; Université de Rennes 1, 2, avenue du Professeur Léon Bernard, F-35043 Rennes (FR). (74) Agents: ABRAMS, Samuel, B. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GH, GW, HU, ID, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: TYPE XVIII COLLAGEN FOR DETECTING LIVER DISEASES		
(57) Abstract Methods directed to detecting or monitoring pathological liver conditins such as cirrhosis and hepatocellular carcinoma by determining the levels of type XVIII collagen is serum are described. A reagent kit and antibodies useful in carrying out such methods are also presented.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TYPE XVIII COLLAGEN FOR DETECTING LIVER DISEASES

5

This application relates to and claims priority to prior United States Provisional Patent Application No. 60/049,369 filed on June 12, 1997.

10

I. FIELD OF THE INVENTION

The present invention is directed to methods of diagnosing liver diseases. More specifically, the present invention is directed to detecting or monitoring the pathogenesis of liver diseases such as cirrhosis and hepatocellular carcinoma by determining the levels of type XVIII collagen in a patient sample. The present invention is further directed to a reagent kit and antibodies useful in carrying out such methods.

II. BACKGROUND OF THE INVENTION

20

Collagen. Collagen is the most abundant component of the extracellular matrix. The collagen superfamily comprises at least nineteen (19) molecules which are generally the result of three polypeptide chains containing, in their primary sequence, $(-Gly-X-Y-)_n$ repeats which allow for the formation of triple helical domains (van der Rest *et al.*, 1991, *FASEB J.* 5:2814-2823). Characterized by their structure and biological function, specific collagen types arrange within extracellular matrices in precise aggregates, maintaining a delicate equilibrium in specialized tissues.

25

A recently discovered collagen molecule, collagen type XVIII, is a non-fibrillar collagen, and belongs to a unique subgroup of the collagen superfamily; the MULTIPLEXIN (multiple triple-helix domains and interruptions) family. Rehn *et al.*, 1996, *Genomics* 32(3): 436-446; Oh *et al.*, 1994, *Genomics* 19(3):494-499. The structure of mouse genes and partial human cDNA and genomic structures of type XVIII collagen have been characterized and identified in, for example, Rehn *et al.*, 1996, *supra*, Muragaki *et al.*, *J. Biol. Chem.*, 1994, 269(6):4042-4046, and Oh *et al.*, 1994, *supra*. Elucidation of the complete mouse chain revealed it to be homologous with the previously identified type XV collagen located in basement membrane zones. Rehn *et*

35

al., 1994, *Proc. Natl. Acad. Sci USA* 91:4234-4238, Myers *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:10144-10148, and Hagg *et al.*, 1997, *Am. J. Pathol.* 150:2075-2086.

The mouse type XVIII collagen, however, differs strikingly from type XV by the presence of variant polypeptide forms characterized by three N-terminal noncollagenous domains of differing lengths originating from two alternate promoters resulting in the synthesis of either a short or two distinct long amino acid non-collagenous domains. Rehn *et al.*, 1996, *supra*; Rehn *et al.*, 1995, *J. Biol. Chem.* 270:4705-4711.

The non-fibrillar collagens, such as type XVIII collagen, demonstrate frequent interruptions in the triple-helical structures conferring flexibility to the molecules. The presence of collagen type XVIII is demonstrated in organs which are highly vascularized, for example, the liver, lung, kidney, and placenta. Of the three N-terminal polypeptide variants of type XVIII collagen, the long forms of the non-collagenous domain were most highly expressed (Rehn *et al.*, 1995, *supra*). The highest levels of collagen type XVIII expression have been observed in the liver. Type XVIII collagen has also been detected in vascular basement membrane zones in mice.

Collagen and Connective Tissue Disorders. A number of diseases are associated with collagen gene expression. In fibrotic conditions, such as liver cirrhosis, expression of collagen genes are increased as a result of injury to the liver and the resultant cooperation of injured hepatocytes with nonparenchymal cells of the liver. The excessive accumulation of collagen resulting from this injury leads to the impairment of normal functioning of the liver (Kivirikko, 1993, *Ann Med* 25:120-122).

In liver cancer diseases such as hepatocellular carcinoma, collagen breakdown and deposition occurs as a result of the cooperation of neoplastic hepatocytes with nonparenchymal cells of the liver. In such disease, well-differentiated tumor cells proliferate along the preexisting matrix scaffold, preserving the trabecular tissue architecture. As the disease progresses, less differentiated cells appear and hepatocellular carcinoma increase in size. Subsequently, the once well-differentiated trabecular pattern is lost as angiogenesis and remodeling of the extracellular matrix occurs.

Methods For Diagnosing Liver Diseases. Current methods of diagnosing and detecting liver disease include the assay of urinary peptide-bound hydroxyproline, determination of hydroxyproline concentration in liver tissue, and serum prolyl

hydroxylase activity. Each of these methods may require biopsies and/or histological examination of the liver tissue, and therefore, are an extremely invasive means of diagnosing and detecting a liver disease.

5 More recently, it has been speculated that measurements of serum levels of the carboxy terminal peptide of type I procollagen may be used to determine the progress of collagen deposition. Nittsu *et al.*, 1988, *Journal of Gastroenterology and Hepatology* 3:159-167. Support for this method for detecting and diagnosing diseases related to collagen deposition can be found in the observations that collagen levels in human body
10 tissues and fluids alter as a result of disease. Type XVIII collagen, however, has not been previously described as a marker for detecting liver diseases.

General Diagnostic Methods For Detecting Diseases. Methods wherein a monoclonal antibody is utilized to diagnose diseases have been described generally. *See*,
15 *e.g.*, United States Patent No. 5,298,393 and United States Patent No. 4,628,027. Specific diagnostic methods utilizing antibodies to specific markers have also been disclosed. For example, the method of diagnosing diseases, such as cancer, utilizing a monoclonal antibody recognizing glutathione S-transferase is described by United States
20 Patent No. 5,298,393. Likewise, monoclonal antibodies which purportedly diagnose fibrosis by recognizing connective tissue proteins, specifically, collagen types I, II, III, IV, and V, are described generally in U.S. Patent No. 4,628,027 ("027 Patent"). The '027 patent, however, does not disclose antibodies specific to type XVIII collagen or their application to the diagnosis of disease.
25

III. SUMMARY OF THE INVENTION

The present invention is based on the discovery that a distinct type of collagen, type XVIII collagen, varies in its distribution throughout body tissues and fluids as result
30 of disease, specifically, in the liver. The present invention provides for various approaches for detecting and measuring the changes in distribution and levels of type XVIII collagen, thereby providing for a diagnostic assessment of the pathological stages of a liver disease associated with type XVIII collagen such as liver cirrhosis and
35 hepatocellular carcinoma.

It is found that type XVIII collagen is abundant in the extracellular matrix of the perisinusoidal space which is associated with the basement membranes in human liver. Its main cellular sources are hepatocytes, endothelial cells and myofibroblasts. Notably, *it situ* hybridizations demonstrated that hepatocytes contain marked amounts of $\alpha 1(\text{XVIII})$ collagen chain mRNAs, suggesting that the strong expression of type XVIII collagen in the liver is mainly due to the synthesis of type XVIII collagen by hepatocytes. In liver diseases associated with fibrosis such as liver cirrhosis, a two-fold increase in $\alpha 1(\text{XVIII})$ mRNA compared to normal liver was detected. Type XVIII collagen expression in liver diseases associated with cancer such as hepatocellular carcinoma, was detected in tumor hepatocytes, the surrounding capsule and in blood vessels.

Methods. The present invention comprises a means of diagnosing liver diseases. Specifically, the present invention provides for the detection and monitoring of the pathogenesis of liver diseases. More specifically, present invention provides for diagnosing liver diseases by measuring the levels of type XVIII collagen in a patient sample, preferably, a patient's serum. The present invention contemplates that type XVIII collagen is released into body fluids under some physiological or pathophysiological conditions, particularly, in fibrosis and in cancer. Determination of levels of type XVIII collagen or degradation products thereof in body fluids such as serum may therefore be diagnostically and prognostically valuable.

In one embodiment of the present invention, a method is provided for the detection and/or monitoring of liver cirrhosis. The present invention provides for measuring the changes of type XVIII collagen from normal liver to liver cirrhosis by detecting the levels of type XVIII collagen with immunohistological and immunoserological techniques.

In another embodiment of the present invention, a method is provided for the detection and/or monitoring of hepatocellular carcinoma facilitating the prognosis of hepatocellular carcinoma. More specifically, a method is provided for measuring the levels of type XVIII collagen in hepatocellular carcinoma with immunohistological and immunoserological techniques, thereby, monitoring the development of the disease.

In a further embodiment of the present invention, a method is provided for the measurement of type collagen XVIII levels in a non-diseased serum sample. Comparison of the levels of type collagen XVIII of serum samples from a patient known or suspected to have a liver disease to a non-diseased serum sample serves as an indicator of a liver disease. The method provides that higher levels of type XVIII collagen from serum samples from patients suspected or known to have a liver disease to levels in non-diseased serum samples is indicative of the presence of liver fibrosis. Higher levels of type XVIII collagen from serum samples of patients suspected or known to have a liver disease to levels in non-diseased serum samples serves as a prognostic indicator of hepatocellular carcinoma.

The method of the present invention utilizes an antibody. The antibody can be a monoclonal or polyclonal antibody capable of specifically detecting type XVIII collagen. In a preferred embodiment of the present invention, the antibody is human or humanized. The preferred antibodies may be used in standard radioimmunoassays or enzyme-linked immunosorbent assays or other assays which are known to utilize antibodies for measurement of levels of type XVIII collagen in a serum sample. The method of utilizing an antibody to measure the levels of type XVIII collagen allows for a non-invasive diagnosis of the pathological states of liver diseases associated with fibrosis and cancer such as liver cirrhosis and hepatocellular carcinoma.

The present invention also provides for the specificity and reliability in detecting and measuring type XVIII collagen in tissue and body fluids for the diagnostic evaluation of the presence and stages of liver diseases associated with fibrosis and cancer, particularly, liver cirrhosis and hepatocellular carcinoma. The present invention provides for methods of diagnosing the presence of liver fibrosis in patients. In addition, the present invention provides for methods of determining the progress of hepatocellular carcinoma in patients, therefore, serving as a prognostic indicator of the extent of the disease.

Diagnostic Kit. The present invention is further directed to a kit consisting of antibodies and reagents. In a preferred embodiment, the diagnostic kit comprises reagents for measuring type XVIII collagen existing in body fluids such as serum. In one embodiment of the present invention, the kit comprises an immobilized antibody

which specifically recognizes collagen type XVIII and an enzyme labeled antibody specific for collagen type XVIII capable of binding to an antigen component different from the immobilized antibody. The kit also comprises the reagents necessary for the detection of the enzyme-labeled antibody. Other reagents which may be necessary may be added, for example, dissolving agents, cleaning agents and reaction terminators.

In a preferred embodiment of the invention, the kit is packaged and labeled for example, in a box or container which includes the necessary elements of the kit, and directions and instructions on the use of such diagnostic kit.

Antibodies. The present invention further comprises antibodies specific for type XVIII collagen. The antibody can be a polyclonal antibody or a monoclonal antibody, preferably, the antibody is a human or humanized antibody to type XVIII collagen. The antibodies of the present invention may be produced by a method which comprises administering in an immunogenic form at least a natural or synthetic part of a polypeptide corresponding to type XVIII collagen to obtain cells producing antibodies reactive with said polypeptide and isolating the antibody containing material from the organism or the cells.

Monoclonal antibodies of the present invention may be generated, for example, by obtaining a hybridoma that produces an antibody specific to type XVIII collagen by fusing a myeloma cell with antibody-secreting cells of an animal immunized with human type XVIII collagen, culturing said hybridoma and/or a cell line arising therefrom, and harvesting the antibody specific to human type XVIII collagen.

Polyclonal antibodies of the present invention may be generated, for example, by immunizing an animal with type XVIII collagen together or after the addition of a suitable adjuvant, such as Freund's incomplete or complete adjuvant. The animals are bled regularly and the blood obtained is separated into an antibody containing serum fraction.

The antibodies of the present invention include antibodies which specifically detect three N-terminal polypeptide variants of human type XVIII collagen, and antibodies specific for the two long N-terminal polypeptide variants of type XVIII collagen. More specifically, antibodies of the present invention specifically detect the long polypeptide variant found virtually in mature liver.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A, 1B, 1C, 1D, and 1E depict the comparative RNA expression of type XVIII collagen, type IV collagen, laminin γ 1, and type XVII collagen, respectively, in normal adult liver in northern blot containing 15 μ g total RNA per lane.

FIGURE 1A depicts RNA expression of type XVIII collagen in normal adult liver.

FIGURE 1B depicts RNA expression of type XV collagen in normal adult liver.

FIGURE 1C depicts RNA expression of laminin γ 1 in normal adult liver.

FIGURE 1D depicts transferred RNA of type XVIII collagen stained with methylene blue.

FIGURE 1E depicts type XVII collagen in the sample samples of normal liver in a Western blot. A distinct band at 215 kDa and a 150-130 kDa doublet are visible.

FIGURE 2A depicts type XVIII collagen expression in normal liver. Immunoperoxidase staining detects type XVIII collagen along perisinusoidal spaces and in basement membrane zones underlying bile ducts (*arrow*). Vascular smooth muscle shows strong staining (*arrowheads*).

FIGURE 2B depicts type XVIII collagen expression in normal liver by *in situ* hybridization with 35 S-labeled cRNA.

FIGURE 2C depicts *in situ* hybridization with 35 S-labeled cRNA showing α 1(XVIII) collagen transcripts mainly in the liver parenchyma.

FIGURE 2D depicts the intense signal in hepatocytes, bile duct epithelium and portal vessels of α 1(XVIII) collagen expression. Portal fibroblasts do not show signal over background noise.

FIGURE 2E depicts the adjacent section hybridized with the α 1(XVIII) collagen sense probe.

FIGURE 3A depicts type XVIII collagen distribution in liver cirrhosis. *In situ* hybridization shows α 1(XVIII) transcripts mainly in cirrhotic nodules.

FIGURE 3B depicts that type XVIII collagen predominates in the cirrhotic nodules shown by immunoperoxidase staining of liver cirrhosis.

FIGURE 3C depicts type XVIII collagen distribution in the cytoplasm of hepatocytes and lining of the sinusoids of liver cirrhosis.

FIGURE 3D depicts expression of type XVIII collagen by endothelial cells in liver cirrhosis.

FIGURE 3E depicts strong expression of type XVIII collagen in ductular structures and surrounding matrix in liver cirrhosis.

FIGURE 4A depicts type XVIII collagen detection in the tumor, the capsule, and the muscular wall of a vessel in hepatocellular carcinoma by immunoperoxidase.

FIGURE 4B depicts expression of endothelial cells of type XVIII collagen in hepatocellular carcinoma by *in situ* hybridization with ³⁵S-labeled cRNA.

FIGURES 4C and 4D depict $\alpha 1(\text{XVIII})$ in the tumor and in stromal cells at the tumor-host interface in hepatocellular carcinoma.

FIGURES 4E and 4F depict intense signal in tumor hepatocytes and endothelium in hepatocellular carcinoma.

FIGURES 5A, 5B, and 5C depict the differential expression of type XVIII collagen in hepatocellular carcinomas.

FIGURE 5A depicts representative cases of expression of the lowest and the highest level of type XVIII collagen RNA expression in hepatocellular carcinoma.

FIGURE 5B depicts two distinct groups of $\alpha 1(\text{XVIII})$ collagen RNA levels in hepatocellular carcinoma.

FIGURE 5C depicts the percent of patients with hepatocellular carcinoma surviving greater than 12 months in both years.

FIGURE 6 depicts cDNA clones encoding the entire $\alpha 1$ chain of human type XVIII collagen and schematic structures of the full-length variant polypeptides of the collagen chains. Collagenous domains are shown in white, non-collagenous domains common to all variants in black, and non-collagenous sequences unique to NC1-493 variant is shown in gray.

FIGURE 7 depicts the alignments of the amino acid residues of the variant-specific regions of the different NC1 domains of human and mouse $\alpha 1(\text{XVIII})$ chains.

FIGURE 8 depicts the amino acid residues of the sequences common to human and mouse $\alpha 1(\text{XVIII})$ collagen chains.

FIGURE 9 depicts the probes used for Northern and *in situ* hybridization analysis and the cDNA fragments used for antigen production and for affinity-purification of the human type XVII collagen specific antibodies.

5 FIGURES 10A, 10B, and 10C depict the characterization of antibodies to human type XVIII collagen by Western Blotting.

FIGURE 10A shows bacterial cell lysates expressing various constructs detected by anti-all huXVIII antibodies.

10 FIGURE 10B shows bacterial cell lysates expressing various constructs detected by anti-long huXVIII antibodies.

FIGURES 11A, 11B, and 11C depict a Northern blot analysis of variant mRNA of human α (XVIII) collagen in several adult and fetal human tissues.

FIGURE 10C shows a Western blot analysis of human kidney protein lysate.

15 FIGURES 11A, 11B, 11C, 11D, 11E, and 11F depict localization of type XVIII collagen mRNAs and protein in human fetal and adult liver.

FIGURES 11A and 11B show a clear hybridization signal in fetal hepatocytes. FIGURE 11A is a light field image and FIGURE 11B is a corresponding dark field
20 image.

FIGURE 11C shows immunostaining of fetal liver with the anti-long huXVIII antibody demonstrating interrupted linear staining along the developing sinusoids. There is strong immunoreactivity around the portal areas and in the BM zone of the blood
25 vessel.

FIGURE 11D shows immunostaining of fetal liver with the anti-long huXVIII antibody demonstrating immunoreactivity only along the developing sinusoidal structures.

FIGURE 11E shows immunostaining of adult liver with the anti-all huXVIII antibody demonstrating linear staining along the sinusoids. The walls of the larger
30 vessels and the BM zones of the bile ducts and capillaries are also strongly stained.

FIGURE 11F shows immunostaining of adult liver with the anti-long huXVIII antibody revealing linear staining along the sinusoids. The staining in the walls of the larger vessels is marked weak, while the BM zones of the capillaries and bile ducts
35 remain negative.

FIGURE 12 identifies type XVIII collagen in human serum by Western Blotting using the anti-long huXVIII and the monoclonal DB144-F1 antibodies. A collagenase sensitive band of 141 kD indicates that a proteolytic fragment derived from the N-terminal part of type XVIII collagen is circulating in serum under normal conditions.

V. DETAILED DESCRIPTION OF THE INVENTION

Antibodies. The present invention provides for antibodies specific for type XVIII collagen. Antibodies of the present invention may be polyclonal or monoclonal antibodies. The antibodies of the present invention may be produced, for example, by a method which comprises administering in an immunogenic form at least a natural or synthetic part of a polypeptide corresponding to type XVIII collagen to obtain cells producing antibodies reactive with said polypeptide and isolating the antibody containing material from the organism or the cells.

Several methods may be employed for obtaining monoclonal antibodies of the present invention. For example, a ready source of monoclonal antibodies may be obtained from fusion of antibody-secreting cells with non-antibody-secreting myeloma cells resulting in a hybridoma. These hybridomas provide a ready source of monoclonal antibodies.

The hybridoma which produces the monoclonal antibodies of the present invention, specifically, the monoclonal antibodies recognizing type XVIII collagen may be produced according to cell fusion techniques as exemplified in publications such as Kohler and Milstein, 1975, *Nature* 256:495-497. In such technique, animals, for example, rabbits, monkeys, mice, rats, goats, etc. are immunized with type XVIII collagen, and antibody producing cells from their spleen, lymph node, etc. are collected and fused with human or animal myeloma cells. The myeloma cell lines that may be used are described in for example, *Monoclonal Antibodies and T-cell Hybridomas in: J.L. Turk (editor) Research Monographs in Immunology Vol. 3, Elsevier/North Holland Biomedical Press, New York (1981)*.

Those hybridomas secreting antibodies of the present invention may then be selected from the colonies of hybridomas produced by cell fusion. Namely, antibodies detecting the three N-terminal polypeptide variants and antibodies detecting the two long

variants of the N-terminal domain are selected. More specifically, antibodies of the present invention specifically detecting the long polypeptide variant found virtually in mature liver. Selection of the desired hybridomas can be conducted by techniques such as enzyme-linked-immunosorbent assay (ELISA) or a radioimmunoassay, as described in
5 *R. Kennet et al, Monoclonal Antibodies, Hybridomas; A New Dimension In Biological Analyses, pp. 376-384, Plenum Press, New York (1980)*, to confirm that the monoclonal antibodies produced by the hybridomas result in an antigen-antibody reaction with type XVIII collagen. The hybridomas secreting the desired monoclonal antibody are cloned
10 into individual antibody-producing cell lines by methods described in, for example, Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York thereby, producing a ready source of monoclonal antibodies specific for type XVIII collagen.

15 Polyclonal antibodies of the present invention may be obtained, for example, by methods as described in Harboe and Ingild, *Scand. J. Immun.* 2 (Suppl.1), 1973, pp. 161-164. More specifically, to obtain polyclonal antibodies of the present invention, animals are injected with a type XVIII compound preparation subcutaneously with complete
20 Freund's adjuvant followed by booster injections with incomplete Freund's adjuvant at certain intervals. The animals are bled regularly, for instance at weekly intervals, and the blood obtained is separated into an antibody containing serum fraction. Optionally, such fraction is subjected to further conventional procedures for antibody purification, and/or procedures involving use of purified type XVIII collagen compounds.

25 The human type XVIII collagen of the present invention consists of short and long variants of $\alpha 1$ (XVIII) collagen chains. The long variants have been found to be expressed virtually in the liver, particularly, in the liver sinusoids. The antibodies of the present invention include, for example, antibodies which detect the short and long
30 polypeptide variants of type XVIII collagen. More specifically, antibodies of the present invention specifically detect the long polypeptide variant found virtually in mature liver.

Methods for obtaining human or humanized antibodies may also be used to obtain antibodies of the present invention. Such methods are described in, for example,
35 European Patent No. EP 765172, European Patent No. EP 671951, United States Patent No. 4,574,116, United States Patent No. 4,350,683, United States Patent No. 5,593,822,

United States Patent No. 5,422,245, United States Patent No. 4,713,351, European Patent No. EP 589877, United States Patent No. 5,565,332, and European Patent No. EP 616640.

5 ***The detection and monitoring of liver diseases.*** As depicted by FIGURES 1A-1D and 2A-2E, type XVIII collagen has been shown to be expressed highly in liver. However, in liver diseases, such as liver cirrhosis, upregulated expression of type XVIII collagen has been detected (FIGURES 3A through 3E). The presence of liver fibrosis, may be detected by the antibodies of the present invention to detect levels of type XVIII
10 collagen expression. For example, serum samples may be withdrawn from patients suspected of having liver cirrhosis and subjected to immunoassays with antibodies against type XVIII collagen. The presence of higher levels of type XVIII collagen compared to normal would be a basis for detecting and monitoring the pathological conditions of liver
15 cirrhosis. The use of antibodies of the present invention provides for an early and accurate diagnosis of liver diseases such as liver cirrhosis.

The detection of type XVIII collagen levels in patients with hepatocellular carcinoma may serve as a prognostic indicator of patient survival. Notably, a negative
20 association between type $\alpha 1$ (XVIII) collagen RNA levels and tumor size and necrosis has been demonstrated. More specifically, the higher type XVIII collagen expressed, hepatocellular carcinoma was found smaller and necrosis infrequent. It has been demonstrated that hepatocellular carcinoma patient survival time is markedly longer in
25 patients who's tumor tissue expresses high amounts of type XVIII collagen compared with low amounts of type XVIII collagen as depicted in FIGURES 5A through 5C. The level of $\alpha 1$ (XVIII) collagen expression may have a prognostic value in hepatocellular carcinoma as the prominent synthesis of type XVII collagen by hepatocytes suggests that
30 it may be secreted into the blood and recruited and activated at sites undergoing angiogenesis.

The antibodies of the present invention may also be used to diagnose pathological conditions of liver diseases by histological diagnosis of tissue samples wherein the antibodies against type XVIII collagen detect changes in distribution of type XVIII
35 collagen. As demonstrated by FIGURES 3A through 3D and 4A through 4F, the pattern of distribution of type XVIII collagen in liver cirrhosis and hepatocellular carcinoma

differs compared to normal liver (FIGURES 2A through 2E). Use of the antibodies specific for type XVIII collagen would allow for detecting changes in type XVIII collagen distribution in liver tissue which could serve as an indicator of the presence or progressive stage of liver diseases, such as liver cirrhosis and hepatocellular carcinoma.

In another aspect of the invention, the antibodies are used to detect and monitor the presence and pathogenesis of a liver disease associated with fibrosis and cancer, specifically, liver cirrhosis and hepatocellular carcinoma. For example, detection of type XVIII collagen in serum samples using antibodies provides for a non-invasive means for diagnosing and monitoring the presence and development of liver diseases such as liver cirrhosis and hepatocellular carcinoma.

Detection of type XVIII collagen levels may be obtained through immunoassay methods, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay or any other known assays which utilize an antibody to detect the presence of a protein marker. The ELISA and radioimmunoassay methods are preferred and may, with the antibodies of the present invention be used to detect specifically the presence of type XVIII collagen. In a preferred method of the invention, serum samples are obtained first from patients suspected or known to have a liver disease. The serum sample is measured for levels of type XVIII collagen through immunoassay, and compared with the amount of type XVIII collagen levels in a second non-diseased serum sample to determine presence or progression of a liver disease. The same methods may be used to monitor a liver disease. For example, a first serum sample measured for levels of type XVIII collagen is obtained from a patient known to have a liver disease, and compared to levels of type XVIII collagen in samples taken subsequent to the first samples. Such method allows for monitoring the progression of a liver disease.

In the present invention, enzyme-linked immunosorbent assay (ELISA) can be used to measure levels of type XVIII collagen present in serum samples. For example, monoclonal antibodies against type XVIII collagen are conjugated to an appropriate enzyme such as horseradish peroxidase, protein ferritin, enzyme alkaline phosphatase, β -D-galactosidase etc. These enzyme-linked antibody preparations are mixed with the serum samples which contain unknown amounts of type XVIII collagen. Since the antibody is specific for type XVIII collagen, antigen-antibody binding occurs after an

incubation period, and transferred to a microtiter plate which have been precoated with type XVIII collagen. Unbound antibodies bind to the antigen absorbed to the walls of the microtiter wells. The initial antigen-antibody complexes are washed out of the wells
5 o the microtiter plate leaving the enzyme-linked antibodies complexed to the antigen coating the walls. A substrate is added and enzymatic activity is measured. The method of enzyme-linked immunosorbent assay (ELISA) is described in *R. Kennet et al.*, 1980, *supra*.

Radioimmunoassay may also be used to measure levels of type XVIII collagen.
10 For example, type XVIII collagen is radioactively labeled and mixed with antibodies specific for type XVIII collagen and a serum sample containing an unknown amount of unlabeled type XVIII collagen. Binding competition between the labeled and unlabeled type XVIII collagen with the antibody occurs. By measuring the amount of radioactivity
15 of the reaction mixture, the amount of type XVIII collagen present in the sample can be quantitatively determined. The method of radioimmunoassay is described further in publications such as US Patent Nos. 4,438,209 and 4,591,573.

The full length human type XVIII collagen cDNA encodes 1516 or 1336 residue
20 $\alpha 1$ (XVIII) collagen chains. Characterization of the primary structure of human type XVII collagen chains has revealed that type XVIII collagen is located ubiquitously in basement membrane zones, its expression pattern being almost identical to the $\alpha 1$ and $\alpha 2$ chains of type IV collagen. Marked differences however were detected in the location of the variant chains. Strikingly, the short and long variant forms (NC1-303 and NC1-493)
25 of type XVIII collagen demonstrate clear tissue specificity in their expression and location in basement membrane zones. The long variant appeared to be virtually the only one present in liver sinusoids. Whereas, the short variant was found in most conventional basement membranes, including those of the blood vessels, the various
30 epithelial structures, and around muscle structures.

The antibodies against type XVIII collagen can be used to detect the short and long polypeptide variants of type XVIII collagen. The predominant cells of the liver, hepatocytes, synthesize specifically the long variants while nonparenchymal cells in liver
35 such as the biliary duct cells and endothelial cells synthesize only the shortest chain variants. Thus, antibodies against the long chain variants can specifically detect type XVIII collagen synthesized by hepatocytes.

Kits. The present invention also involves reagents for measuring type XVIII collagen in fluid samples. The diagnostic kit of the invention contains reagents for measuring levels of type XVIII collagen in serum samples. This reagent kit comprises an antibody which specifically detects type XVIII collagen bound to a support, and a second antibody which is specific to type XVIII collagen that binds to a different epitope of the type XVIII collagen molecule. The second antibody is enzyme-labeled. In addition, the reagent kit of the present invention comprises reagents for detecting the enzyme-labeled antibody. The reagent kit employs immunological methods in measuring type XVIII collagen in the serum sample, thus, allowing for the detection and monitoring of liver diseases, specifically, liver cirrhosis and hepatocellular carcinoma. The kit is packaged and labeled, for example, in a box or container which includes the necessary elements of the kit, and directions and instructions on the use of such diagnostic kit.

The following examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Preparation Of Antibodies Specific For Type XVIII Collagen

Expression of Polypeptide Fragments in E. coli for Antigen Production.

A 700 bp cDNA fragment QH48, corresponding to the common region of the NC1 domain of human type XVIII collagen, was generated by PCR using HL4 as a template and primers H18-HIS-4 and H18-HIS-8 (see below). The fragment was subcloned after KpnI/HindIII digestion into the vector PQ#-41 (Qiagen, Inc., Santa Clarita, CA, USA), which can be used to express in-frame dihydrofolate reductase (DHFR) fusion proteins

with an N-terminal His-tag (clone QH48), and transformed into the *E. coli* strain M15. The QH48 fragment was expressed as suggest by Qiagen, the bacterial culture was pelleted and resuspended in 6 M guanidine/HCL, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9, frozen in -70 °C and lysed at room temperature for 1 hour. The suspension was
5 centrifuged for 20 minutes at 12000 g, the supernatant was lightly sonicated and 5 mM imidazole was added, after it was applied to a 0.75x 5 cm ProBond column (Invitrogen, Leek, Netherlands) pre-equilibrated with 8 M urea, 0.5 M NaCl, and 20 mM Tris/HCl, pH 7.9. The denatured polypeptides bound to the column were allowed to renature by
10 means of a slow stepwise decrease in the concentration of urea for 2 hours in a buffer of 0.5 M NaCl and 20 mM Tris/HCl, pH 7.9. The polypeptides were eluted using a stepwise imidazole gradient from 0 M to 0.5 M NaCl and 20 mM Tris/HCl, pH 7.9. The eluted fractions were monitored at A₂₈₀, and verified by SDS-PAGE and Coomassie
15 staining. The fractions containing the human type XVIII collagen-derived polypeptide were dialyzed against 1x PBS and concentrated by ultrafiltration (MWCO 10 kD, Millipore).

A 430 bp fragment QH67, corresponding to the long NC1 domain, was amplified
20 as above, except that the template was a genomic subclone HE1.2 of the human COL18A1 gene, and the primers were H18-HIS-6 and H18-HIS-7. This PCR product was subcloned into the vector pQE-41, and the clone QH67 was expressed and purified as for QH 48 above.

Antibodies to the fusion proteins QH48 and QH67, were raised by conventional
25 methods. For immunization, 300 µl of purified fusion protein solution was injected into rabbits subcutaneously with complete Freund's adjuvant (Sigma, St. Louis, USA) followed by booster injections with incomplete Freund's adjuvant (Sigma, St. Louis, USA) at intervals of 14 days. The sera were tested by Western blotting using crude
30 bacterial cell lysates from the expressed clones, QH48.18 and QH1415, encoding type XVIII collagen sequences without the DHFR leader sequences. Oligonucleotides used were (KpnI and HindIII restriction sites are underlined): H18-HIS-4: 5' ATAAGCTTA
CGTGGAGACAGATTC 3' ; H18-HIS-8: 5' AT GGTACC CAGCCTCTTCTTCC 3';
35 H18-HIS-6: 5' AT AAGCTT AGGGCCCCGTGAGTGG 3'; and H18-HIS-7: 5' AT GGTACCCCGGAATGGTTCCA 3'.

Affinity Purification and Characterization of Human Type XVIII Collagen-

Specific Antibodies. Two additional bacterial expression constructs were made to aid affinity purification of the sera. The expression clone QH48.18 was the same as QH48, except that the insert subcloned into the vector pQE-31 (Qiagen, Inc., Santa Clarita, CA, USA), which can be used to express polypeptides with an N-terminal His-tag without the DHFR leader. A slightly longer construct than QH67, a 550 bp fragment QH1415.7, was produced using the primers H18-HIS-14 and H18-HIS-15 (H18-HIS-14:

5' ATGGTACCCTGGTTCAATAATGAGGG 3', KpnI and HindIII restriction and subcloned into the vector pQE-31.

The fragments were expressed and purified as above, except that an additional purification step was included for the QH1415.7 fragment. This was dialyzed in 20 mM piperazine, pH 5.5, after the ProBond column and applied to a HiTrap Q anion-exchange column according to the instructions of the manufacturer (Pharmacia Biotech. Inc., Uppsala, Sweden) and the bound polypeptides were eluted with a stepwise gradient of NaCl from 0 M to 1 M in 20 mM piperazine, pH 5.5. The positive fractions of QH48.18 and QH1415.7 following the ProBond or HiTrap Q column, respectively, were pooled and dialyzed in 0.5 M NaCl, 0.2 M NaHCO₃, pH 8.6, after they were coupled to CNBr-Sepharose (Pharmacia Biotech. Inc., Uppsala, Sweden) according to the manufacturer's protocol. The identities of the isolated polypeptide were confirmed by sequencing their N-terminal ends using automated Edman degradation with an Applied Biosystems' model, 477A Protein Sequencer.

For affinity purification of the type XVIII collagen-specific antibodies, the antisera were diluted 1:1 with PBS, pH 7.4, and applied to the respective columns, which were subsequently washed with 2 M NaCl/PBS, pH 7.4. The bound antibody molecules were eluted with 150 mM glycine/HCl, pH 2.5, and then with 100 mM triethylamine, pH 11.0. The fractions containing protein were detected at A₂₈₀, immediately neutralized with 2M Tris/HCl, pH 7.5, pooled and concentrated (MWCO 100 kD, Millipore).

1 ml aliquots of the isopropyl-β-D-thiogalactopyranoside-induced bacterial cell cultures of DHFR, QH48, H48.18, QH67 and QH1415.7 were centrifuged, suspended in 200 µl of 20 mM NaPO₄, pH 7.2, and treated for analysis under reduced conditions by 12% SDS-PAGE followed by staining with Coomassie Brilliant Blue and Western

blotting onto nitrocellulose filters. The affinity-purified antibodies were used at dilutions of 1 µl of 20 mM NaPO₄, pH 7.2, and treated for analysis under reduced conditions by 12% SDS-PAGE followed by staining with Coomassie Brilliant Blue and Western blotting onto nitrocellulose filters. The affinity-purified antibodies were used at dilutions of 1 µg/ml for 2 hours at room temperature and detected with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) and Enhanced Chemiluminescence detection reagents (Amersham Corp., Buckinghamshire, UK) as recommended. The antibodies were also characterized by using a kidney tissue sample. Thirty µg of Human Kidney Protein Medley sample (Clontech, Palo Alto, USA) with 5% 2 mercaptoethanol was boiled for 5 minutes, applied to a 7% SDS-PAGE and treated for Western blotting using the type XVIII collagen antibodies. The immunosignals were detected using ECL as above.

Preparation, Affinity Purification and Characterization of Two Polyclonal Antibodies to Human Type XVIII Collagen. The construct QH48, corresponding to all type XVIII variants, and QH67, corresponding to the long variant, were expressed as DHFR fusion proteins in *E.coli*. (FIGURE 9) and the purified recombinant proteins used to immunize rabbits. The ensuing sera were affinity-purified using type XVIII polypeptide fragments expressed without the DHFR leader sequences in order to isolate type XVIII specific antibodies designated as anti-all huXVIII and anti-long huXVIII antibodies.

The affinity-purified antibodies were first characterized using crude bacterial cell lysates expressing variant polypeptide sequences. The anti-all huXVIII antibody was tested using the DHFR construct as a negative control and QH48 and QH48.18 fragments (the latter lacking the DHFR sequence) as positive ones. No immunosignal was visible in the DHFR sample as expected whereas QH48 and QH48.18 gave specific signals of 52 kD and 32 kD, respectively, demonstrating that the anti-all huXVIII antibody is capable of detecting type XVIII polypeptides. In the case of the anti-long huXVIII antibody, no immunosignal was visible using the DHFR sample, whereas 44 kD and 33 kD bands were detected with the QH67 and QH1415.7 constructs, respectively, (the latter lacking the DHFR sequences). Thus, the anti-long huXVIII antibody detected polypeptide sequences derived from the long type XVIII variants. A minor 32 kD band was also

detected in the QH1415.7 sample, which was most probably a degradation product from QH1415.7 since it was not detected in the DHFR or QH67 samples.

Both antibodies were further characterized using a human kidney protein lysate in Western blotting. Adult human kidney contains marked amounts of mRNAs for the short XVIII variant and much lower amounts of mRNAs for the long variant (FIGURE 10).

The two antibodies gave overlapping specific results, as expected. The anti-all huXVIII antibody resulted in a broad, smear-like band above the highest 200 kD molecular weight standard, as is typical of proteoglycans containing long GAG side chains. Since type

XVIII collagen chains contain several potential sites for the attachment of GAG chains, it is tempting to propose that, at least in the human kidney, type XVIII is in a proteoglycan form. As the kidney sample was a commercial one already homogenized in high

concentration of SDS, we were not able to perform digestions of sugar chains to further

characterize the nature of the side chains. Interestingly, the anti-long huXVIII resulted in a similar smear-like signal, but with less intensity. Moreover, the signal with the anti-

long huXVIII antibody overlapped the one obtained with the anti-all huXVIII antibody, but consisted only of the upper part of the broad smear detected with the latter, a finding

consistent with the expected polypeptide lengths of the long and short variants. Also, the intensities of the bands were in agreement with the results obtained by

immunohistochemistry where the anti-long huXVIII gave far weaker immunosignals than the anti-all huXVIII. These results suggest that the two antibodies can be used to detect human type XVIII collagen in a specific manner.

One notable finding was the lack of staining for the NC1-493 variant in capillaries and only weak staining in the walls of large arteries (FIGURE 11D),

compared with strong signals with the anti-all huXVIII antibody (FIGURES 11A, 11C, and 11E). Only the vascular smooth muscle structures contain the long variant, since

there is staining in the walls of the larger vessels in all tissues studied, but not in other smooth muscle structures such as the smooth muscle cells in the dermis or in the

bronchial walls. The strong staining seen with the anti-all huXVIII antibody in the epithelial BM zones of the bile ducts, kidney tubules, pancreatic acini and ducts, lung

alveoli and bronchial structures, and in the basement membrane zones of the Bowman's capsule and peripheral nerves was lacking the anti-long huXVIII antibody.

B. Example 2: Type XVIII Collagen Expression In Normal Liver

The expression of type XVIII collagen was studied on normal liver tissues by northern blot, western blot, immunohistochemistry and *in situ* hybridization. All specimens were routinely processed for histology, *i.e.*, hematoxylin-eosin-saffron and Sirius red staining.

To study type XVIII collagen expression by northern blot, after histological analysis, frozen blocks making up a volume of 1.5 to 3 cm were used for RNA extraction by the guanidinium thiocyanate/cesium chloride method. Histological analysis of the frozen block before RNA extraction from large tissue samples ensured that sampling was both macroscopically and microscopically consistent. About 500 μ g to 1 mg RNA was obtained per sample with a purity as assessed by OD₂₆₀/OD₂₈₀ ratio of 1.7 to 2.0 RNA integrity was assessed by ethidium bromide staining after separation of 5 μ g RNA on a 1.2% denaturing agarose gel. Only samples with a 28S/18S ratio > 2/1 and without contaminating DNA were included in the study. For northern blots, 15 μ g RNA was run in a 1.2% denaturing agarose slab gel and transferred onto a Hybond N nylon membrane (Amersham, Buckinghamshire, UK). After UV-crosslinking the efficiency of transfer was assessed by methylene blue staining. cDNA probes were labeled to high specific activities (1x10⁸ to 1x10⁹ cpm/ μ g cDNA) using the Rediprime™ kit (Amersham) and α -dCTP ³²P 3000 Ci/mmol (Amersham). Hybridizations with the cDNA probes were performed in 0.5 M NaPO₄ containing 7% SDS 3x 1 min and 2x 15 min at 65 °C. Amersham Hyperfilm-MP films were exposed with enhancing screens at -80 °C. To achieve a visual comparison between the mRNA levels of C XVIII with collagen α 1 (IV) and laminin γ 1 mRNA levels in normal liver, the same filter was subsequently hybridized and stripped; and autoradiography exposure was for 48 hr for all probes.

By northern blot, multiple α 1(XVIII) collagen transcripts were identified, varying in size between 4.5-7.4 Kb, with two major bands at about 5.0 and 6.0 Kb (FIGURE 1A). α 1(XVIII) collagen RNA level was several-fold higher than those of collagen α 1(IV) and laminin γ 1 in these samples (FIGURES 1A through 1D).

Collagen type XVIII was detected in samples of normal liver by western blot. For protein extraction, 5- μ m cryostat sections making up surface of 1 cm² were freeze-dried on microscope slides and scrapped off with scalpel blades. The tissue scrapings

were homogenized in 10% SDS, 1% β -mercaptoethanol, 10 mM Tris/HCl pH 6.8 and 20% glycerol. 40 μ g protein was electrophoresed in a 7.5% polyacrylamide slab gel and electroblotted overnight onto Hybond ECL nitrocellulose membranes (Amersham). After blocking the filters in 3% BSA-PBS, they were incubated with peroxidase-conjugate, goat-anti-rabbit IgG. All incubations were done at room temperature for 1 hr.

Peroxidase activity was developed by enhanced chemiluminescence (ECL Western blotting detection system, Amersham). By western blot, two polypeptide bands at 215 kDa and 150 kDa were identified in the same samples. The 150 kDa band sometimes resolved as a 130, 150 kDa doublet (FIGURE 1E).

Expression of type XVIII collagen was also studied by immunohistochemistry. 5 μ m cryostat sections were fixed in 4% paraformaldehyde buffered in PBS (pH 7.4) and blocked in 2% BSA-PBS (w/v) for 1 hr. All antibody incubations were done at room temperature for 1 hr and antibody dilution performed in 2% BSA-PBS. *Single labeling:* Serial sections were incubated with specific anti collagen XVIII or IV rabbit IgG. After washes in PBS, sections were incubated with peroxidase-conjugate, goat-anti-rabbit IgG. Sections were lightly counterstained with hematoxylin. Control staining was performed with non-immune rabbit IgG at the same dilution used for the primary antibodies.

Double labeling: Serial sections were simultaneously incubated with anti collagen XVIII rabbit IgG and either monoclonal anti- α -SM-1 or with monoclonal anti-human von Willebrand Factor. After washes in PBS, they were simultaneously incubated with both TRITC-conjugate, goat-anti-rabbit IgG and FITC-conjugate, sheep-anti-mouse IgG in the presence of 5% normal sheep serum (Sigma). Slides were rinsed in PBS and mounted in glycerol-DABCO. Control staining was performed with non-immune rabbit IgG and the secondary antibodies alone. Specimens were observed with an Olympus BX60 microscope equipped for reflected light fluorescence observation. For TRITC, the dichroic mirror was DM 570, the excitation wavelength transmitted by the exciter filter was 530-550 nm, and the barrier filter was BA 590. For FITC, the dichroic mirror was DM 505, the excitation wavelength transmitted by the excitation filter was 460-490 nm, and the barrier filter was BA 515-550. Under these conditions, the partial superposition of the emission spectra of the two fluorochromes was negligible. The detection of endothelial cells or myofibroblasts expressing collagen XVIII was achieved by double exposure.

By immunohistochemistry, type XVIII collagen was strongly detected in the perisinusoidal space, in the basement membranes underlying bile ducts and in vascular smooth muscle cells (FIGURE 2A). The extracellular matrix of portal tracts showed minimal staining. *In situ* hybridization with sense and antisense cRNA probes was performed. Briefly, linearized expression vectors carrying the α I (XVIII), α I (IV) collagens or laminin γ I inserts were used for *in vitro* transcription in the presence of 60 mCi ^{35}S rUTP (1,250 Ci/mmol) (Amersham). 5 μm frozen sections were fixed in 4% paraformaldehyde, permeabilized and acetylated. Hybridization was carried out overnight at 50 °C with 8×10^3 cpm/ μl . After stringency washes, RNase A digestion and dehydration, slides were exposed to ILFORD G5 emulsion (Ilford, Lyon, France) at 4°C, for 2 weeks for C XVIII and 3 weeks for collagen α 1 (IV) and laminin γ 1. Microautoradiographies were stained with hematoxylin and eosin. Brightfield and darkfield images were obtained with an Olympus BX60 microscope and UPlanFi objectives. For darkfield images, optimization with an Olympus PM20 exposure control unit showed that a 2-step underexposure resulted in the optimal signal-to-noise ration for α I (XVIII), α I (IV) collagens and laminin γ I probes. Optimized autoradiography and darkfield microphotography allow objective comparison of RNA levels in the different tissue compartments in the iconography presented.

In situ hybridization demonstrated a high expression of α 1(XVIII) collagen RNA in the liver parenchyma, particularly within hepatocytes (FIGURES 2B through 2D). In portal tracts, bile duct epithelium and vascular endothelial and smooth muscle cells were also heavily decorated with α 1(XVIII) collagen RNA.

Study of normal liver tissue demonstrated that type XVIII collagen in liver, specifically in the perisinusoidal space, and that it is associated with basement membranes in human liver. In the liver, the main cellular sources for type XVIII collagen are hepatocytes, endothelial cells, and myofibroblasts.

C. Example 3: Type XVIII Collagen Expression In Liver Cirrhosis

The distribution of type XVIII collagen in liver cirrhosis was analyzed by immunohistochemistry techniques described in Example 2. By *in situ* hybridization and immunoperoxidase, collagen XVIII was distributed in cirrhotic nodules (FIGURES 3A

and 3B) with a low signal in the mature connective tissue of fibrous septa (FIGURES 3A through 3C). Hepatocytes were packed with collagen XVIII, which formed a thick lining along capillarized sinusoids (FIGURE 3D). Collagen XVIII delineated basement
5 membrane structures underlying ductular hepatocytes and proliferating bile ducts. Myofibroblasts embedded in this remodeling immature connective tissue expressed collagen XVIII (FIGURES 3E and 3D). By double immunofluorescence, vWF-positive endothelial cells along peripheral sinusoids of regenerating nodules were intensely stained for collagen XVIII (FIGURE 3D).

10 A two-fold increase in mRNA for type XVIII collagen in liver cirrhosis compared to normal liver was detected. A strong signal in hepatocytes and along the sinusoids of type XVIII collagen expression was demonstrated. In addition, smooth muscle, peripheral nerves, and capillarized sinusoids in liver cirrhosis demonstrated thick deposits
15 of type XVIII collagen.

D. Example 4: Type XVIII Collagen Expression In Hepatocellular Carcinoma

20 Type XVIII collagen expression in hepatocellular carcinoma ("HCC") was studied in tissues samples from 21 male, and 1 female, the mean $\pm$ SD age was 64.5 $\pm$ 7.8 years HCC patients. HCC tumor size was distributed as follows: 5HCC were $\leq$ 2 cm, 4 were > 2 to 5 cm, 5 were > 5 to 10 cm and 8 were > 10cm. Ten tumors displayed predominantly trabecular (plastelike) pattern, 12 were pseudoglandular (acinar).

25 Edmonson's score was = II in 17 cases; III in 4 cases and IV in one case. All HCC arose in fibrotic livers. Eleven HCC had portal immobilization and 9 were encapsulated. Microscopic foci of tumor necrosis were found in 8 cases. Follow-up data were available for 18 patients, mean $\pm$ SD survival time was 20.7 $\pm$ 16.14 months, (range = 4 to
30 68). Localization of type XVIII collagen was studied using a combination of immunoperoxidase, double immunofluorescence staining and *in situ* hybridization, the techniques of which are presented in Example 2, *supra*. Tumor hepatocytes and endothelial cells were the main cellular sources of collagen type XVIII in HCC (FIGURES 4A through 4F). Stromal myofibroblasts expressed moderate amounts of
35 collagen XVIII (FIGURE 4C). The expression of α 1(XVIII) collagen RNA by tumor stromal cells closely resembled that of α 1(IV) collagen and laminin γ 1 RNA's. Collagen

XVIII expression was variable in HCC. In cases with trabecular histological pattern, the tumor cells were packed with collagen XVIII, whereas in cases with pseudoglandular (acinar) features, collagen XVIII was restricted to vessels and stromal cells with a lower signal in tumor cells.

Northern and dot blot analysis $\alpha 1(\text{XVIII})$ collagen RNA levels demonstrated a highly variable $\alpha 1(\text{XVIII})$ collagen RNA expression characterized the HCC population (FIGURES 5A and 5B) (mean $\pm$ SD = 1.7 ± 1.2 , minimum = 0.15; maximum = 5.6) with a 37-fold difference between the case expressing the highest and the lowest RNA levels and without significant difference between HCC and control liver.

Using a clustering algorithm, 2 distinct groups of $\alpha 1(\text{XVIII})$ RNA values were discerned (FIGURE 6C): CXVIII high showed a 3-fold higher mean RNA level than the group CXVIII low (mean $\pm$ SD: CXVIII high = 2.7 ± 1.2 , 95% confidence interval = 1.81-3.59, n = 10; CXVIII low = 0.9 ± 0.4 , 95% confidence interval = 0.63-1.16, n=11; $p<0.001$). CXVIII high were significantly different from normal livers ($p<0.001$) whereas CXVIII low were not. These differences were confirmed by northern blot (FIGURE 5A) and, at the protein level, by immunohistochemistry. Moreover, 85.7% of CXVIII high patients survived more than 12 months. Notably, only 27.2% of CXVIII low patients survived more than 12 months.

E. Example 5: Characterization of Type XVIII Collagen

Isolation of cDNAs and Characterization of Alternative Splicing.

A 455 bp cDNA fragment was synthesized from human placenta cDNA by PCR using the oligonucleotides RY-1 and RY-2 corresponding to the C-terminal noncollagenous sequences of the mouse $\alpha 1(\text{XVIII})$ cDNA chain (RY-1, 5'ATGAATTTCGGAGCAGTTTC/TCAG/ATGT/CTT 3'; RY-2, 5'ATGAATTCATGAAGCTG/ATTC/TTCT/A/GATA/GCA 3'. RY-1 and RY-2 cover nucleotides 3495-3514 and 3929-3948, respectively, as previously described Rehn *et al.*, 1994, *supra*, and bases added to generate a complete EcoRI restriction site, together with two base extensions, are underlined. The 455 bp fragment was used to screen a human placenta λ gt11 cDNA library (HL 1075b, Clontech, Palo Alto, USA), and the ensuing

positive recombinant phages were isolated and subcloned into an EcoRI site of Bluescript SK (Stratagene, La Jolla, CA, USA).

A cDNA library was constructed from MG63 osteosarcoma cell mRNA using the oligonucleotide JR2-RT (5' CTCGGTCTCCCTTCTCC 3') as a human $\alpha 1$ (XVIII) collagen-specific primer and the Time-Saver-cDNA synthesis kit (Pharmacia Biotech, Inc., Uppsala, Sweden). The cDNAs were ligated into a λ gt10 vector (Stratagene, La Jolla, CA, USA) and packed into bacteriophage λ particles using the *in vitro* packaging extract (Amersham Corp., Buckinghamshire, UK). This library was screened at low stringency with an AccI fragment of the clone ME-1, which covers sequences extending from the NC1 to the COL6 domain of mouse type XVIII collagen (Rehn and Pihlajaniemi 1995, *supra*), resulting in isolation of the clone L16A, which was in turn used to screen a human fetal liver cDNA library (HL 1064a, Clontech, Palo Alto, USA), leading to the isolation of several recombinant phages. A synthetic oligonucleotide was derived from the 5' end of one of these clones, FL7.1.1, and used as a probe to screen a human adult kidney cDNA library (HL1123b, Clontech, Palo Alto, USA), from which the recombinant clone HK16.2 was recovered. A 100 bp EcoRI-AvaI insert of the 5' end of HK16.2 was used to screen a human adult liver cDNA library (HL 1115b, Clontech, Palo Alto, USA) resulting in the isolation of clones HL4 and HL8, and 166 bp EcoRI-ApaI fragment from the 5' end of HL4 was used as a probe to re-screen the human liver cDNA library, resulting in the isolation of a clone HuL8.2. A 264 bp EcoRI-ApaI insert from the 5' end of the HuL8.2 was used to screen the same library, the resulting clone Hli12.1 being obtained by PCR from the λ -DNA and characterized as above. A 254 bp cDNA fragment corresponding to 5' sequences of the common region of the NC1 domain was synthesized by PCR from HL4 using the oligonucleotides LVPCR-5' (5'-GGGCTGCTGCAGCTCCTTGGGGAC-3') (SEQ ID NO:4) and LVPCR-3' (5'-CCAGAGAGCTTCACGCCAGCAAG-3') (SEQ ID NO:5) and used to screen a human fetal kidney cDNA library (HL 5004b, Clontech, Palo Alto, USA), resulting in the isolation of clone HFK1.1.

To study further the alternative splicing detected in HL4, PCR was carried out with primers flanking the spliced area, resulting in either 264 bp or 393 bp cDNA fragments corresponding to the spliced or intact form of the human $\alpha 1$ (XVIII) mRNA,

respectively. The primers used were :18SPL-1: 5'-TGGAGGCAGCAGCACGGA-3',
18SPL-2: 5'-GTCGCCCCGGCTTTCCGTC-3'. The template cDNAs were synthesized
from human fetal liver total RNA with random hexamers and from human HT-1080 cell
line, jejunum and skin RNAs with an oligo(dT) primer.

Nucleotide sequences were determined for both strands of all cDNAs by the
dideoxynucleotide method as described in for example, Sanger, *et al.*, 1977, *Proc. Natl.*
Acad. Sci. USA 74:5463-5467, using T7 polymerase. DNASIS and PROSIS (Pharmacia,
Biotech. Inc., Uppsala, Sweden) were used to analyze the nucleotide and amino acid
sequence data. Nucleotide and amino acid comparisons were made against the Genbank,
EMBL, PIR and SWISSPROT databases using the BLAST network service. The signal
peptide sequences were predicted using the SignalP V1.1 World Wide Web Prediction
Server as described in, for example, Nielsen *et al.*, 1997, *Protein Engineering* 10:1-6.

The search for structural motifs was carried out with the PROSITE database.

Isolation of Human cDNA Clones Encoding The $\alpha 1$ (XVIII) Collagen Chain. A

455-bp PCR fragment corresponding to the C-terminal noncollagenous region of the
mouse $\alpha 1$ (XVIII) collagen chain was synthesized from human placenta cDNA and used
to screen a human placenta cDNA library. Among the numerous positive clones, the two
covering most sequences were the 1.56-kb clone HP19.3 and the 1.72-kb HP12.4, the
latter including two polyadenylation signals 1.15 kb apart (FIGURE 6). Because all the
cDNA clones obtained from the human placenta cDNA library ended at the same
collagenous region as HP19.3, a cDNA library was constructed from MG63 osteosarcoma
cell mRNA with and oligonucleotide specific to the human $\alpha 1$ (XVIII) chain cDNA,
resulting in identification of the 0.68 kb clone L16A use of this clone to screen a human
fetal liver cDNA library resulted in the isolation of FL7.1.1. Subsequently, a synthetic
oligonucleotide was derived from the extreme 5' end of the FL7.1.1 cDNA clone and
used as a probe to screen a human fetal kidney cDNA library, resulting in the isolation
of HK16.2 A 0.1 kb DEcoRI-AvaI insert of HK16.2 was used to screen a human adult
liver cDNA library, and although the ensuing 2.1 kb clone HL8 did not contain any new
information, the 1.2 kb clone, HL4, extended 0.7 kb further upstream. It was detected
that HL4 lacked 129 nucleotides encoding the first collagenous domain and a part of the
NC2 domain as compared with previously characterized cDNA clones. The same cDNA

library was screened with a 0.17 kb EcoRI-ApaI insert derived from HL4 resulting in isolation of clone HuL8.2, the 5' end of which extended 0.35 kb upstream from HL4. The human adult liver cDNA library was re-screened with a 0.26 kb EcoRI-ApaI insert from the 5' end of HuL8.2 and a 1 kb clone Hli12.1, extending 0.2 kb further in the 5' direction was obtained.

In order to obtain a human cDNA clone corresponding to the NC1-301 variant (short variant) of mouse type XVIII collagen, a 254-bp PCR fragment from HL4 was used to probe a human fetal kidney cDNA library. A 0.46 kb clone, HFK1.1, was found covering the sequences specific to the short variant of the human type XVIII collagen. Human cDNA clones corresponding to the mouse NC1-746 $\alpha 1$ (XVIII) collagen chain variant characterized by a cysteine-rich sequence as described in, for example, Muragaki *et al.*, 1995, *supra*; Rehn and Pihlajaniemi, 1995, *supra*) were not found despite extensive screening of cDNA libraries, nor by PCR, suggesting that this variant occurs only in minor amounts in man.

F. Example 6: Expression of Type XVIII Collagen Variants

Nucleotide and Amino Acid Sequences for Two Variant Human

$\alpha 1$ (XVIII) Chains. The sequences of the human cDNA clones can be translated into 1336 residue or 1516 residue polypeptides with predicted molecular weights of either 136 or 154 kD, respectively. Prediction of the signal peptide cleavage sites as described in, for example, Nielsen, *et al.*, 1997, *supra*, suggested that the 1336 amino acid variant contains a 33 residue signal peptide and the 1516 amino acid variant a 23 residue one (FIGURES 6 and 7).

The putative signal peptide of the short variant is followed by a 303 residue noncollagenous domain with only two residues specific to this variant and 301 common to both the short and long variants (FIGURES 6, 7, and 8). The common region contains two conserved cysteine residues also found in the mouse polypeptide and has a 200 residue sequence that is homologous with an N-terminal segment of thrombospondin-1, a multifunctional glycoprotein with affinity for a number molecules as described in Adams and Lawler, 1993, *Curr. Biol.* 3:188-190. This motif has previously been identified in the N-terminus of the mouse type XVIII and XV collagens and in

several other collagen types (Bork, *et al.*, 1992, *FEBS Lett.* 307:49-54; Wälchli *et al.*, 1993, *Eur. J. Biochem.* 212:483-490; Kivirikko *et al.*, 1994, *supra*; Rehn and Pihlajaniemi 1994, *supra*; Hägg *et al.* 1997, *supra*). The length of the N-terminal noncollagenous region of the long variant is 493 residues, excluding the putative signal peptide, which is followed by 192 residues specific to this variant. The NC1 domains of the two forms of the polypeptides may thus be termed NC1-303 and NC1-493.

The collagenous sequence is 688 residues in length and consists of ten collagenous domains (COL1-COL10) varying in length from 18 to 122 residues (FIGURES 6 and 8) and separated by nine noncollagenous domains (NC2-NC10) of between 12 and 26 residues. Two cysteine residues are present in this central region, located in the NC2 domain. Furthermore, five of the collagenous domains contain interruptions of two to five residues in their collagenous sequence. The C-terminal noncollagenous domain (NC11) is 312 residues in length and contains four cysteine residues.

Searches for potentially significant motifs in the human $\alpha 1$ (XVIII) polypeptide sequence led to the identification of four putative sites for N-linked glycosylation, three of which were located in the NC1 domain of the NC1-493 variant and one in the COL3 domain (FIGURES 6 and 8). Two sequences conforming to a consensus sequence for the attachment of glycosaminoglycan (GAG) side chains were found in the NC1 and NC3 domains (FIGURES 6 and 8), and the COL8 domain was found to contain one Arg-Gly-Asp sequence that may play a role in cell attachment. Ruoslahti, 1991, *Integrins J. Clin. Invest.* 87:1-5.

Expression of Variant Type XVIII RNA. Northern blots containing poly(A) + RNA isolated from various human adult and fetal tissues were hybridized with an NC1-303-specific probe HFK.1.1-E/A, resulting in signals of 4.5 kb and 5.6 kb, while an NC1-493-specific probe HuL8.2-E/N recognized two bands of 5.0 and 6.2 kb (FIGURES 9 and 10). All these signals were seen with the probe HL8, covering sequences common to both human $\alpha 1$ (XVIII) chain variants (FIGURE 10).

Of the adult tissues, the probe common to all $\alpha 1$ (XVIII) variants showed strongest hybridization signals with liver RNA (FIGURE 10). This signal was also seen with HuL8.2-E/N, while hybridizations with HFK1.1-E/A remained negative, suggesting

that the type XVIII collagen RNA signal in liver is mainly derived from the long form of the collagen. The intensive signals seen in heart, placenta, pancreas, ovary and small intestine RNAs, the moderate signals in skeletal muscle, kidney, spleen, prostate and testis RNAs, and the weak signals in colon, lung and thymus RNAs were mostly due to hybridization to the NC1-303 variant RNAs. The strongest signals in the fetal tissues were seen in the kidney and liver. The kidney signal was mainly due to the NC1-303 variant RNA, as were the faint signals seen in the fetal lung and brain mRNA. The fetal liver showed strong hybridization for the NC1-493 variant, but also slight hybridization for the NC1-303 variant.

Detection of Serum Form of Type XVIII Collagen by Immunoprecipitation.

The polyclonal anti-long huXVIII antibody detecting long variants of type XVIII and the monoclonal antibody, DB144-F1, detecting all type XVIII variants were used in

immunoprecipitation of normal human serum sample to detect type XVIII collagen. The

monoclonal, DB144-F1, antibody was produced using the fragment QH48, *supra*. For

detection of the serum form of type XVIII collagen, 100 µl of sheep anti-rabbit M-280 Dynabeads were coated with 10 µg of affinity-purified anti-long huXVIII. The control

beads were coated with an irrelevant affinity-purified antibody detecting mouse type

XIII collagen. After washing of excess antibody, 250 µl of normal human serum was

incubated with the beads at 4 °C for overnight, washed several times with 1% Triton X-100/PBS, pH 7.4, and then equilibrated several times with 0.1% BSA, 5 mM CaCl₂, 50

mM Tris/HCl at pH 7.2. 20 U of chromatographically purified bacterial collagenase

(Worthington Biochemical Corp., Freehold, NJ) was added to the reaction volume of 1

ml, and incubated with rotation at +37°C for 4 hours. The control digestions were

incubated in same conditions, but without the added enzyme. The beads were then

washed with 1% Triton X-100-0.1% SDS-PBS, pH 7.4 for several times, suspended in

40 µl of 1x SDS-sample buffer-5% b-mercaptoethanol and boiled for 5 minutes. The

supernatants were removed from the beads, and one immunoprecipitation sample was divided into two Western samples analyzed by denaturing 7% SDS-PAGE, and blotted

onto nitrocellulose filters. The anti-long huXVIII and the DB144-F1 antibodies were

used at dilutions of 1.5 µg/ml and 2 µg/ml, respectively, for 2 hours at room temperature

and detected with a horseradish peroxidase-conjugated goat anti-rabbit or secondary

antibody (Bio-Rad, CA, USA) and Enhanced Chemiluminescence detection reagents (Amersham Corp., Buckinghamshire, UK).

As demonstrated in FIGURE 12, immunoprecipitation samples corresponding to 125 µl of human serum analyzed by Western blotting, both anti-long huXVIII and DB144-F1 antibodies resulted in a 150 kD signal, which was also sensitive to bacterial collagenase. The control antibody used in immunoprecipitations gave no signals. The 141 kD signal corresponds to the liver-specific type XVIII variant, since it was immunoprecipitated using the anti-long huXVIII antibody. The size of the full-length variant expressed in COS-1 cell is 210 kD which suggests that the signal detected from serum is the proteolytically cleaved fragment of type XVIII.

The two independent antibodies detected the same 150 kD polypeptide in human serum. Since the long variant is synthesized at huge amounts in liver and only at minor amount in certain other tissues indicating that the 150 kD band represents type XVIII collagen synthesized by the liver.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety. In addition, the publications listed below are of interest in connection with various aspects of the invention and are incorporated herein as part of the disclosure:

CLAIMS

The following is claimed:

- 5 1. A method for detecting a pathological liver condition in a patient suspected of having a liver disease comprising:
 - a. reacting a first sample from said patient with an antibody specific for type XVIII collagen wherein said patient is suspected of having a liver disease;
 - 10 b. allowing said antibody specific for type XVIII collagen to interact to form an antigen-antibody complex;
 - c. measuring the amount of said antigen-antibody complex to determine the amount of said type XVIII collagen present in the reaction mixture;
 - d. repeating each of steps a, b, and c on a second non-diseased control serum sample;
 - 15 e. comparing the amount of type XVIII collagen in first sample to the amount of type XVIII collagen in second sample to determine presence or progression of said liver disease.
- 20 2. The method for detecting a pathological liver condition according to claim 1 wherein said antibody specific for type XVIII collagen is enzyme-labeled and the amount of said antigen-antibody complexes formed in each of the first and second sample mixtures are measured by:
 - 25 a. removing uncomplexed enzyme-labeled antibodies from each of said first and second sample mixtures by allowing said uncomplexed enzyme-labeled antibody-antigen complexes to form on a surface by contacting said mixtures with such surface to which type XVIII collagen is bound;
 - 30 b. removing of enzyme-labeled antibody-antigen complexes from said surface; and
 - c. contacting the enzyme-labeled antibody complexed to said surface-bound type XVIII collagen with a substrate of the enzyme, measuring the enzyme activity to determine the amount of type XVIII collagen in the first and
35 second samples.

3. The method according to claim 2 wherein the sample from a patient suspected to have a pathological liver condition contains higher levels of type XVIII collagen compared to levels in the control non-diseased sample is indicative of a liver disease.

5

4. The method according to claim 3 wherein said liver disease is cirrhosis.

5. The method according to claim 2 wherein the sample from a patient suspected to have a pathological liver condition contains higher levels of type XVIII collagen compared to levels in the control non-diseased serum sample is predictive of the prognosis of a liver disease.

10

6. The method according to claim 5 wherein said liver disease is hepatocellular carcinoma.

15

7. The method of detecting a pathological liver condition according to claim 1 wherein a known amount of radiolabeled type XVIII collagen is added after the antibody specific for said type XVIII collagen has been added to the first and second samples and the amount of antigen-antibody complexes formed in each said samples is measured by:

20

- a. adding a preparation of anti-immunoglobulin to each mixture to form immune complexes with the antigen-antibody complexes;
- b. separating the immune complexes from supernatant fractions of the mixtures, measuring the radioactivity of the immune complexes or the supernatant fractions to determine the amount of type XVIII collagen in the first or second samples.

25

30

8. The method according to claim 7 wherein the sample from a patient suspected to have a pathological liver condition contains higher levels of type XVIII collagen compared to levels in the control non-diseased sample is indicative of a liver disease.

35

9. The method according to claim 8 wherein said liver disease is cirrhosis.

10. The method according to claim 7 wherein the sample from a patient
5 suspected or known to have a liver disease contains higher levels of type XVIII collagen
compared to levels in the control non-diseased sample is predictive of the prognosis of a
liver disease.

11. The method according to claim 10 wherein said liver disease is
10 hepatocellular carcinoma.

13. The method according to claim 1 wherein said sample is a serum sample.

14. The method according to claim 1 wherein said antibody specific for type
15 XVIII collagen is a monoclonal or polyclonal antibody.

15. The method according to claim 1 wherein said antibody specific for type
20 XVIII collagen is a human or humanized antibody.

16. A method for monitoring a pathological liver condition in a patient known
to have a liver disease comprising:

- 25 a. reacting a first sample from said patient with an antibody specific
for type XVIII collagen wherein said patient is known of having a
liver disease;
- b. allowing said antibody specific for type XVIII collagen to interact
to form an antigen-antibody complex;
- 30 c. measuring the amount of said antigen-antibody complex to
determine the amount of said type XVIII collagen present in the
reaction mixture;
- d. repeating each of steps a, b, and c on a second sample of said
35 patient taken subsequent to first sample over a period of time ;

- e. comparing the amount of type XVIII collagen in first sample to the amount of type XVIII collagen in second sample to determine presence or progression of said liver disease.

5

17. The method for monitoring a pathological liver condition according to claim 16 wherein said antibody specific for type XVIII collagen is enzyme-labeled and the amount of said antigen-antibody complexes formed in each of the first and second sample mixtures are measured by:

10

- a. removing uncomplexed enzyme-labeled antibodies from each of said first and second sample mixtures by allowing said uncomplexed enzyme-labeled antibody-antigen complexes to form on a surface by contacting said mixtures with such surface to which type XVIII collagen is bound;
- 15 b. removing of enzyme-labeled antibody-antigen complexes from said surface; and
- c. contacting the enzyme-labeled antibody complexed to said surface-bound type XVIII collagen with a substrate of the enzyme, measuring the enzyme activity to determine the amount of type XVIII collagen in the first and
- 20 second samples.

20

25

18. The method according to claim 17 wherein the sample from a patient suspected or known to have a pathological liver condition contains higher levels of type XVIII collagen compared to levels in the control non-diseased sample is indicative of a liver disease.

30

19. The method according to claim 18 wherein said liver disease is cirrhosis.

35

20. The method according to claim 17 wherein the sample from a patient suspected or known to have a liver disease contains higher levels of type XVIII collagen compared to levels in the control non-diseased serum sample is predictive of the prognosis of a liver disease.

21. The method according to claim 20 wherein said liver disease is hepatocellular carcinoma.

5 22. The method of monitoring a pathological liver condition according to claim 16 wherein a known amount of radiolabeled type XVIII collagen is added after the antibody specific for said type XVIII collagen has been added to the first and second samples and the amount of antigen-antibody complexes formed in each said samples is measured by:

- 10 a. adding a preparation of anti-immunoglobulin to each mixture to form immune complexes with the antigen-antibody complexes;
- b. separating the immune complexes from supernatant fractions of the mixtures, measuring the radioactivity of the immune complexes or the supernatant fractions to determine the amount of type XVIII collagen in
- 15 the first or second samples.

23. The method according to claim 22 wherein the sample from a patient suspected or known to have a liver disease contains higher levels of type XVIII collagen compared to levels in the control non-diseased sample is indicative of a liver disease.

20

24. The method according to claim 23 wherein said liver disease is cirrhosis.

25 25. The method according to claim 22 wherein the sample from a patient suspected or known to have a liver disease contains higher levels of type XVIII collagen compared to levels in the control non-diseased sample is predictive of the prognosis of a liver disease.

30 26. The method according to claim 25 wherein said liver disease is hepatocellular carcinoma.

35 27. The method according to claim 16 wherein said sample is a serum sample.

28. The method according to claim 16 wherein said antibody specific for type XVIII collagen is a monoclonal or polyclonal antibody.

5 29. The method according to claim 16 wherein said antibody specific for type XVIII collagen is a human or humanized antibody.

30. A reagent kit comprising:

- 10 a. a first antibody that specifically binds to collagen type XVIII bound to a support;
- b. a second antibody that specifically binds to collagen type XVIII that binds to an epitope different from the first antibody wherein said second antibody is enzyme-labeled; and
- 15 c. reagents for detecting enzyme-labeled antibody.

31. A reagent kit for detecting or monitoring the pathogenesis of liver diseases comprising:

- 20 a. a first antibody that specifically binds to collagen type XVIII bound to a support;
- b. a second antibody that specifically binds to collagen type XVIII that binds to an epitope different from the first antibody wherein said second antibody is enzyme-labeled.
- 25 c. reagents for detecting enzyme-labeled antibody.

32. The reagent kit according to claim 14 wherein said liver diseases are liver cirrhosis or hepatocellular carcinoma.

30 33. An antibody that specifically binds to collagen type XVIII.

34. The antibody of claim 33 which specifically detects the short and long N-terminal polypeptides variants of type XVIII collagen.

35

35. The antibody of claim 33 which specifically detects the long N-terminal polypeptide variants of type XVIII collagen.

5 36. The antibody of claim 33 wherein said antibody is a monoclonal or polyclonal antibody.

37. The antibody of claim 33 wherein said antibody is a human or humanized antibody.
10

15

20

25

30

35

FIG. 1A

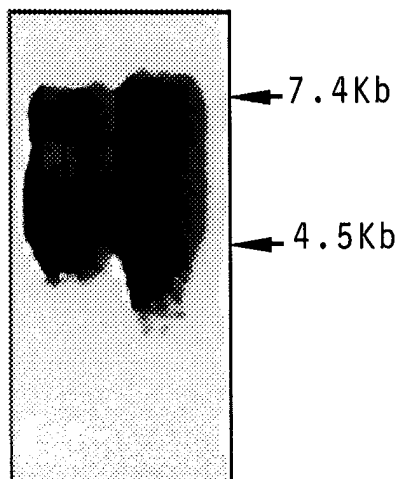


FIG. 1B

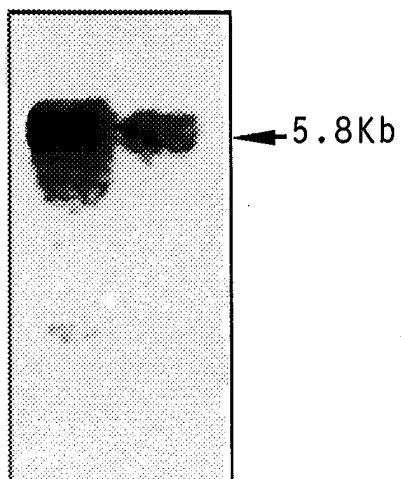


FIG. 1C

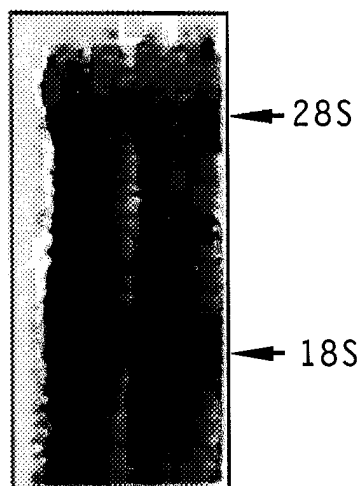
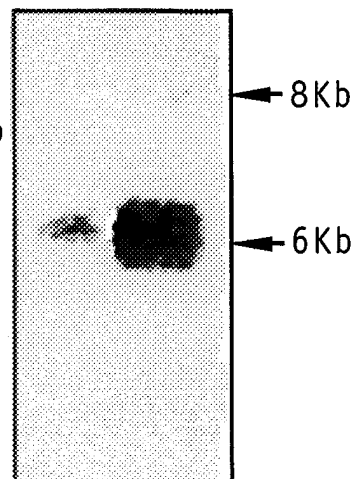


FIG. 1D

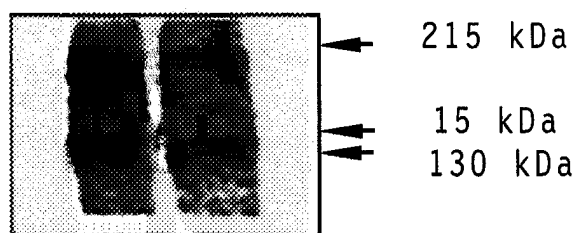


FIG. 1E

FIG. 2B

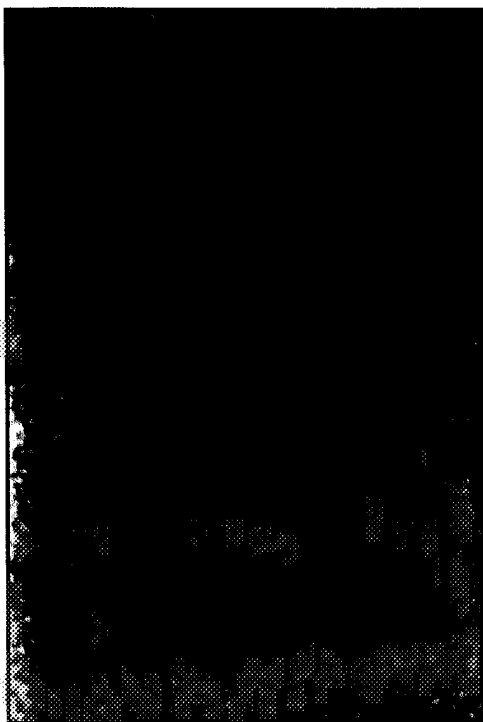


FIG. 2A

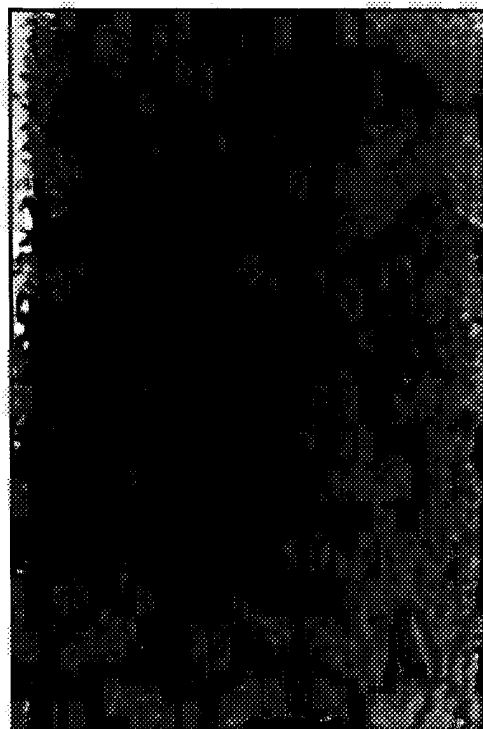


FIG. 2E



FIG. 2D



FIG. 2C

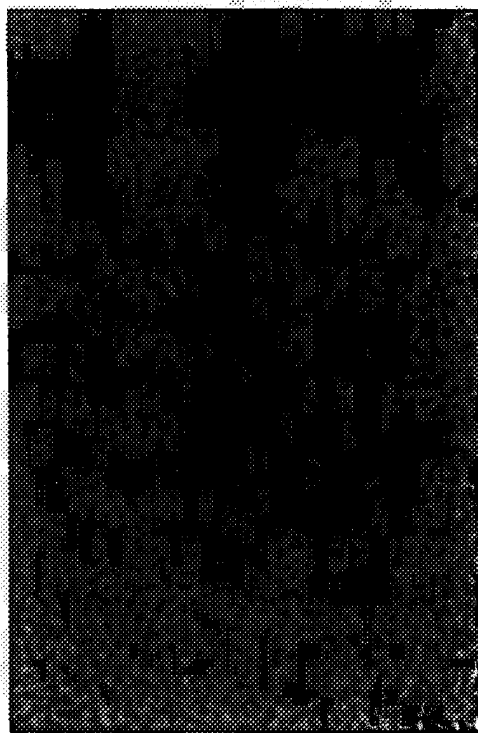


FIG. 3A

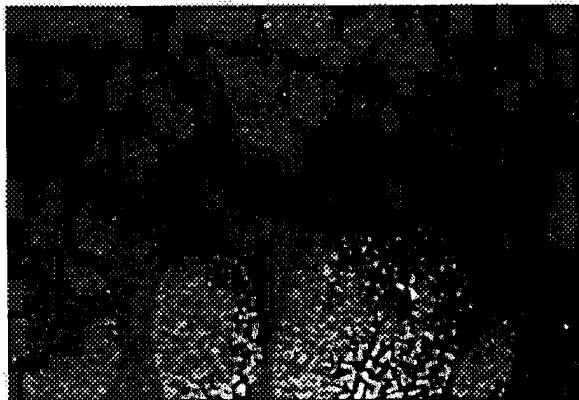


FIG. 3B

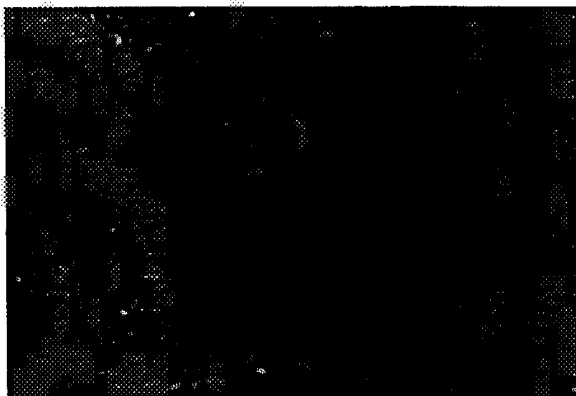


FIG. 3C

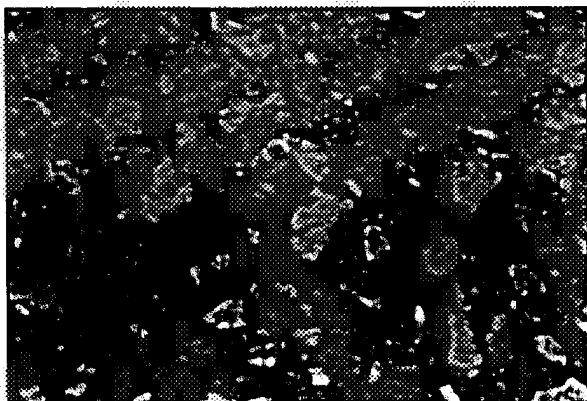


FIG. 3D



FIG. 3E

FIG. 4A



FIG. 4B

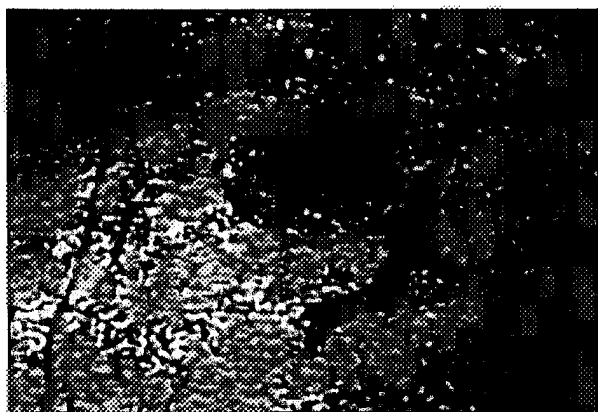
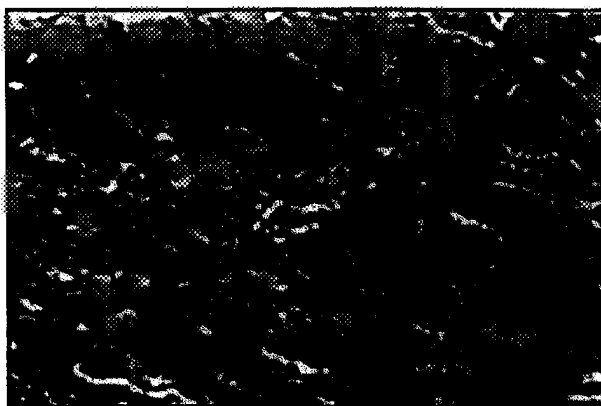


FIG. 4C

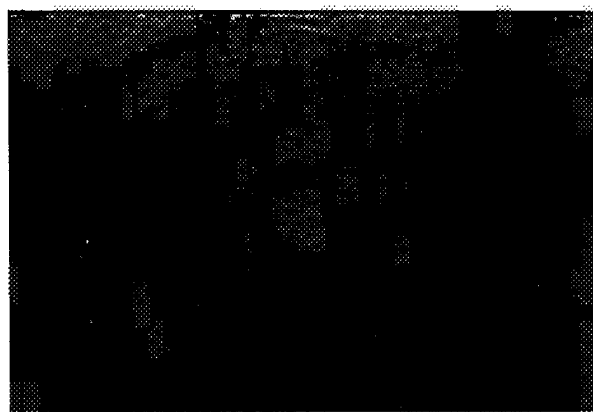


FIG. 4D



FIG. 4E

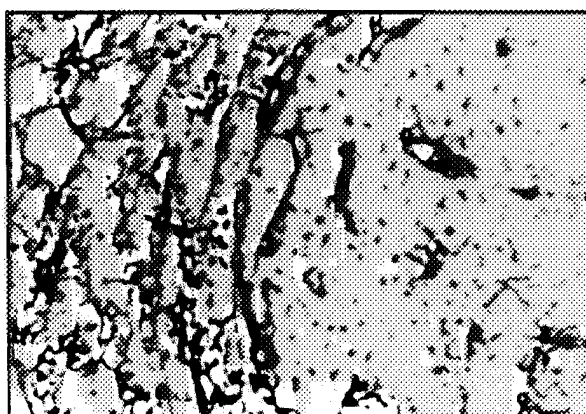


FIG. 4F

5/14

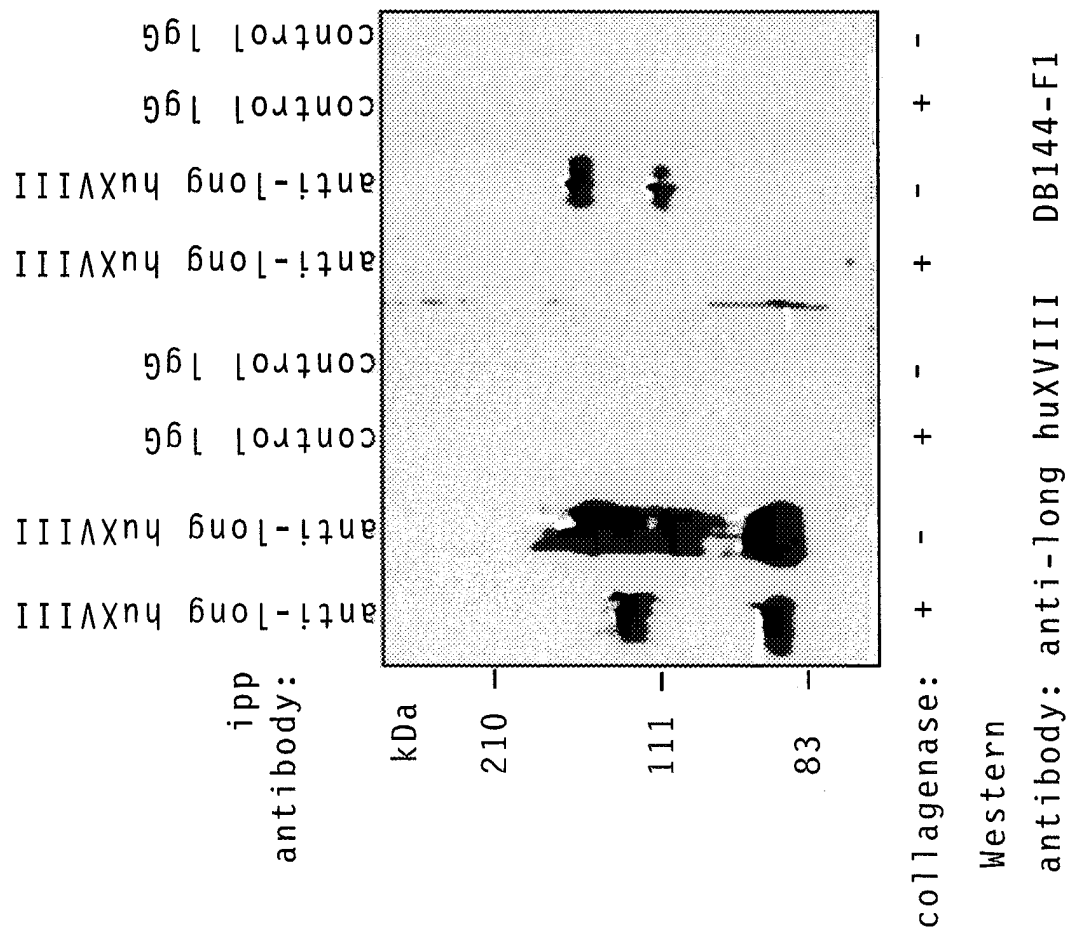
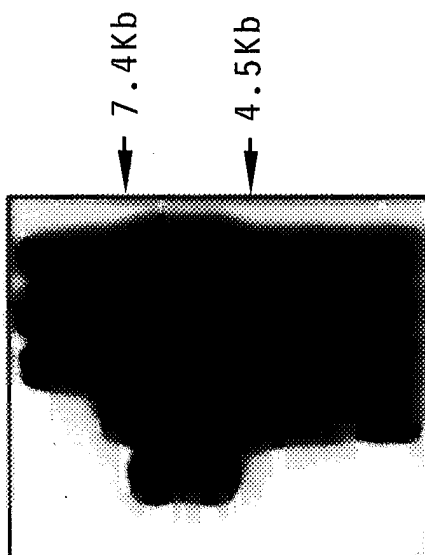


FIG. 12

FIG. 5A



6/14

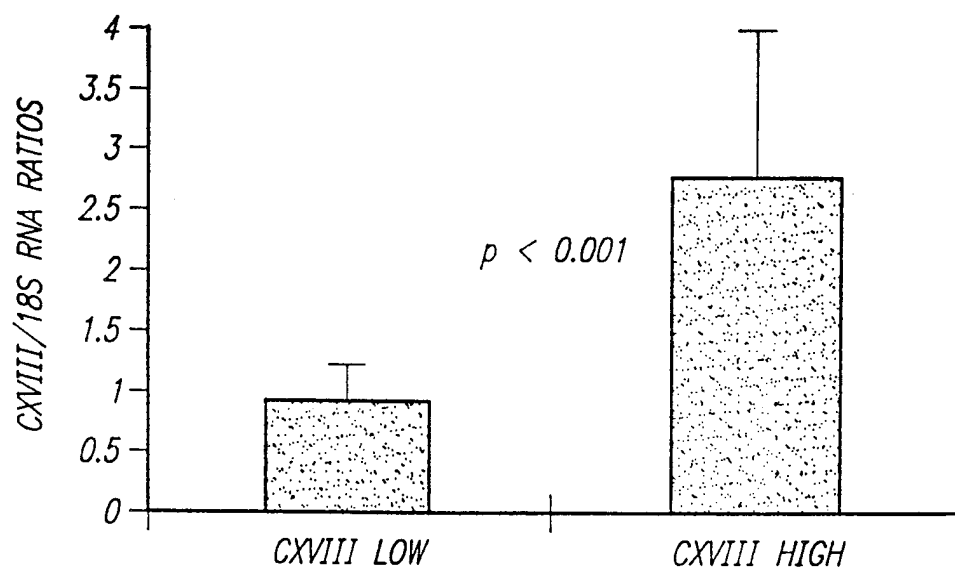


FIG. 5B

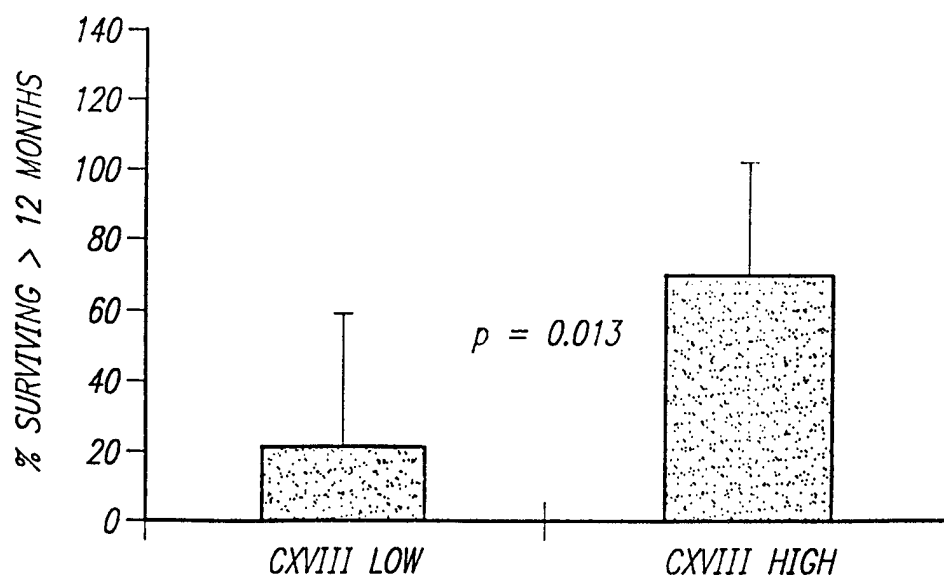


FIG. 5C

7/14

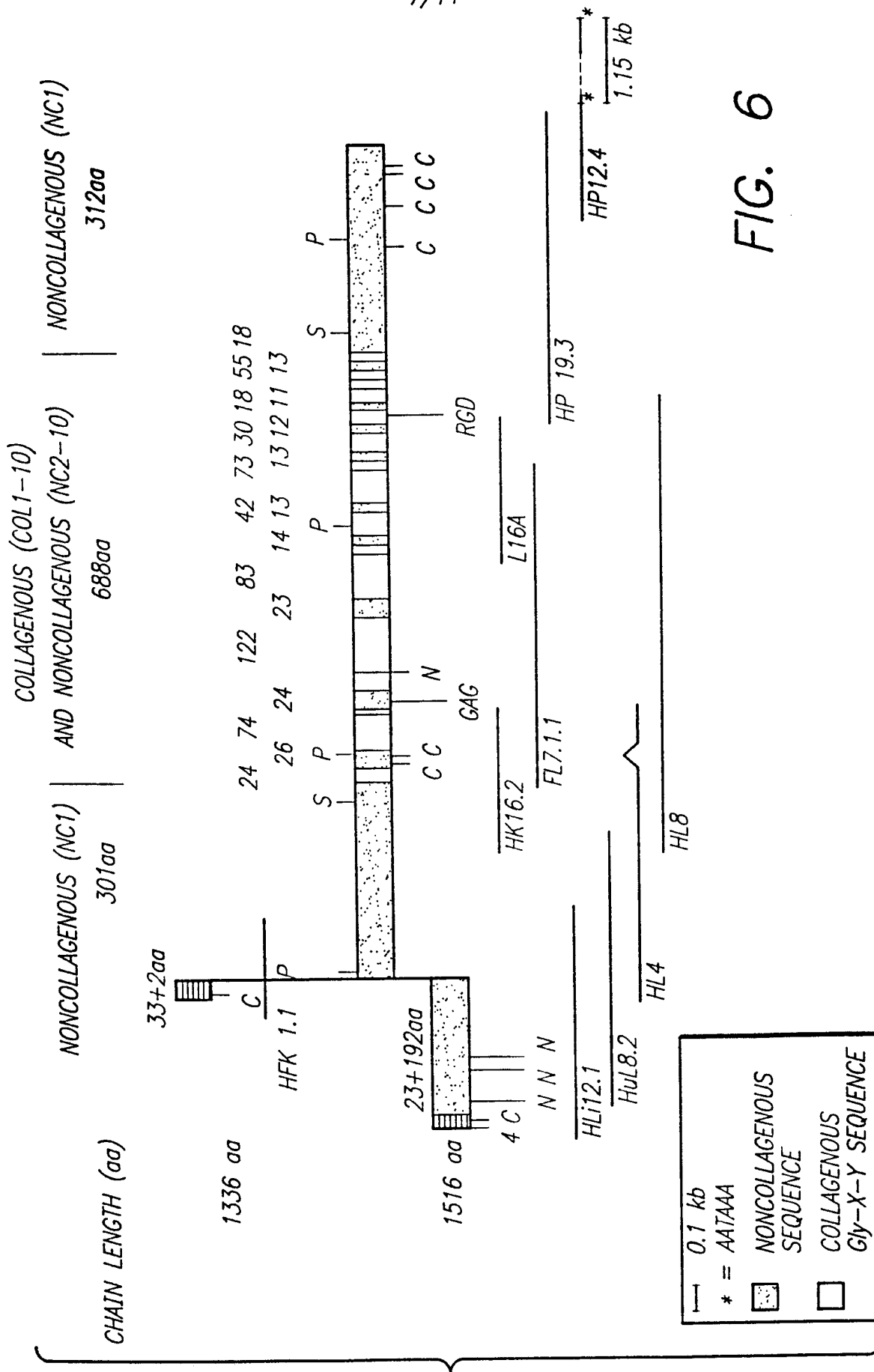


FIG. 6

FIG. 8A

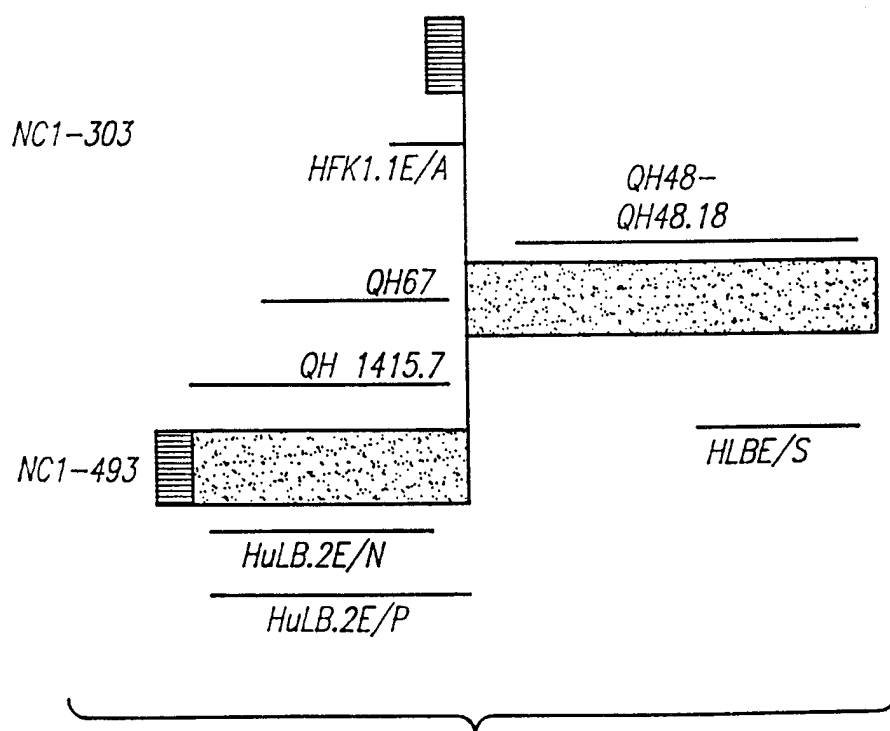
NC1	ERISEEVGLQLLGDPPPPQVTQDDPDVGLAYVFGPDANSQVARYHFP	SLFFRDFSLLFHIRPATEGVLF	FAITDSAQAM
36	ERISEEVGLQLLGDPPPPQVTQDDPDVGLAYVFGPDANSQVARYHFP	SLFFRDFSLLFHIRPATEGVLF	FAITDSAQAM
MO18 (common)	ENVAEEVGLQLLGDPLPEKISQIDDPHVGPAYIFGPDNSGVAQVYHFP	PKLFFRDFSLLFHVRPATEAAGVLF	FAITDAAQV
NC1	ENVAEEVGLQLLGDPLPEKISQIDDPHVGPAYIFGPDNSGVAQVYHFP	PKLFFRDFSLLFHVRPATEAAGVLF	FAITDAAQV
119	VLLGVKLSGVQDGHQDISLLYTEPGAGQTHTAASRLPAFVGQWTHLALSVAGGFVALYVDCEEFRMPLARSSRGLELEPGAGL	FVAQAGGA	
111	VSLGVKLSVRDGGQINISLLYTEPGASQTQTASRLPAFVGQWTHFALSVDDGGSVALYVDCEEFRVVPFARASQGLELERGAGL	FVGAGTA	
212	DPDKFQGVIAELKVRDPQVSPMHCLDEEGDDSDGAFGDSGSLGDARELLREETGAALKPRLPAPPPVTTPLAGGSSTEDSRSEEVEEQTT		
204	DPDKFQGMISELKVRKTRPVSPVHCLDEEDDEDASGDFGSGFEESKSHKEDT--SLLPGLPQPPVTSPLAGGSTTEDPRTEETEDAA		
305	VASLGAQTLPGSDSVSTWDGSRVTPGGRVKEGGLKGKQGE	PGVP	GGPGRAGPPGSPCLPGPPGLPCVSP
295	VDSIGAETLPGTGSSGAWDEAIQNPGRLIKGGMKGQKEFGA	QGGPPGAGPQG	-----PAG---
398	DGTPGRDGE	PGDPGEDGKPGDTPGQFGPTGPDVGP	KDGDPGVGERGPPPGPPGPPGSPFRHDKLTFIDMEGSGFGGDLEALRGPRGPF
378	DGTPGRDGE	PGDPGEDGRPDTGPQFGPTGPDVGP	KDGDPGIGPRGP-----GPPGPPGSPFRQDKLTFIDMEGSGFGSGDIESLRGPRGPF
491	GPPGPPGVPLPGEPRGFVNSSDVP	GPAGLPGVPGREGPPFGPLPGPPPGREGPPGRTGQKSLGEAGAPGHKSGKAGPAGARGESG	
468	GPPGPPGVPLPGEPRFGTNGSVAP	GPAGLPGVPGKEGPPFGPPGPPGPPGPPGKEGPPGVAGQKGSVDVGIPGPKSGDLP	GI

10/14

FIG. 8B

[illegible]

12/14



13/14

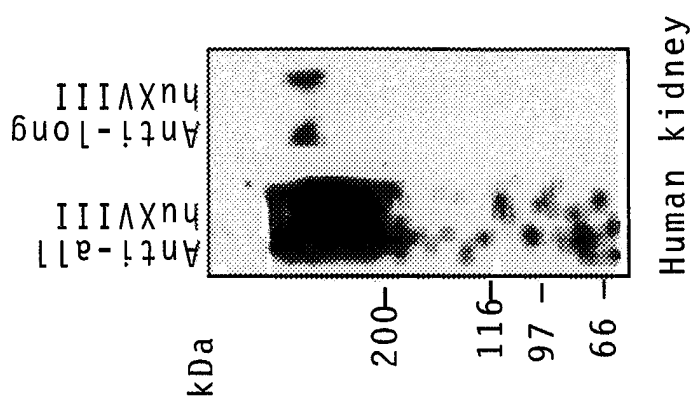


FIG. 10C

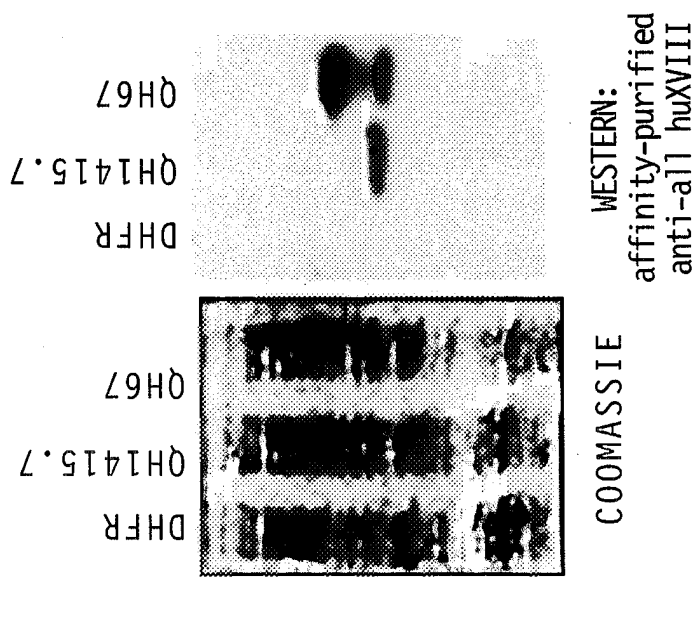


FIG. 10B

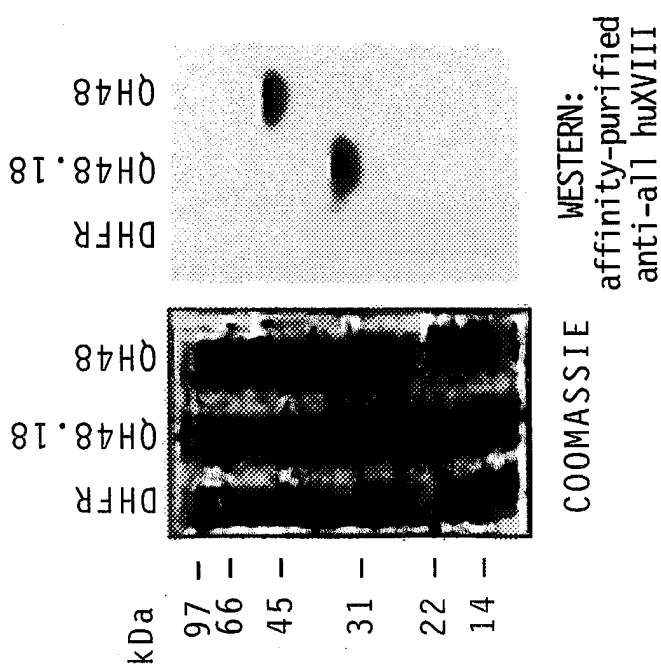


FIG. 10A

FIG. 11A

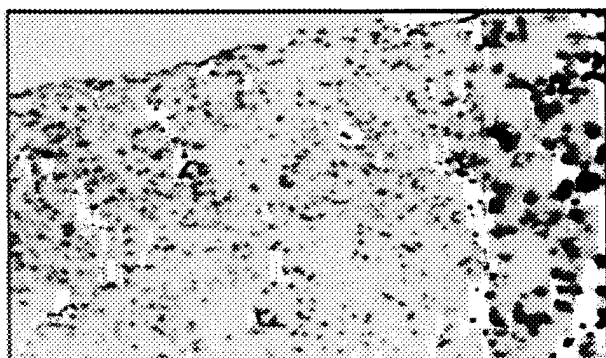


FIG. 11B

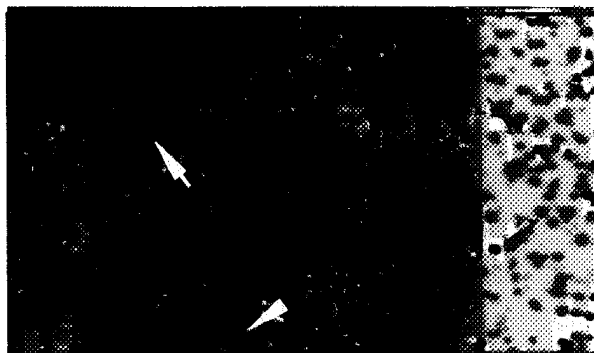


FIG. 11C

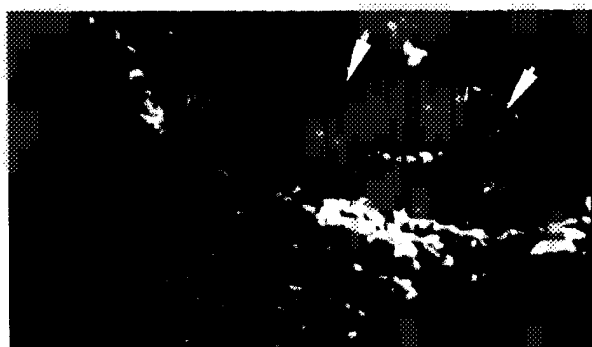


FIG. 11D

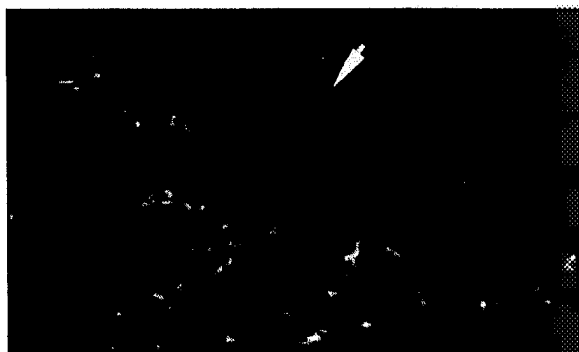


FIG. 11E



FIG. 11F



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/12327

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

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. : 435/7.1, 7.2, 7.8, 7.92, 7.93, 7.94, 7.95; 436/518, 811, 813; 530/388.8, 388.85, 389.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

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

Medline, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,643,783 A (OLSEN et al) 01 July 1997, see entire document.	1-37
Y	OH et al. Isolation and Sequencing of cDNAs for Proteins with Multiple Domains of Gly-Xaa-Yaa Repeats Identify a Distinct Family of Collagenous Proteins Proceedings Of The National Academy Of Sciences, USA. 10 May 1994, Vol 91, No. 10, pages 4229-4233, see entire document.	1-37

☒ 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		
*E	earlier document published on or after the international filing date	*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
*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)	*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
*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	*G	document member of the same patent family

Date of the actual completion of the international search

19 AUGUST 1998

Date of mailing of the international search report

08 SEP 1998

 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

JEFFREY STUCKER

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12327

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	REHN et al. α 1 (XVIII), a Collagen Chain with Frequent Interruptions in the Collagenous Sequence, a Distinct Tissue Distribution, and Homology with Type XV Collagen. Proceedings Of The National Academy Of Sciences, USA. 10 May 1994, Vol. 91, No. 10, pages 4234-4238, see entire document.	1-37
Y	MURAGAKI et al Mouse Coll18a1 is Expressed in a Tissue-Specific Manner as Three Alternative Variants and is Localized in Basement Membrane Zones Proceedings Of The National Academy Of Sciences, USA. 12 September 1995, Vol. 92, No. 19, pages 8763-8767, see entire document.	1-37

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/12327

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 38/00; C07K 5/00, 7/00, 16/00, 17/00; G01N 33/53, 33/537, 33/542, 33/543, 33/567

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/7.1, 7.2, 7.8, 7.92, 7.93, 7.94, 7.95; 436/518, 811, 813; 530/388.8, 388.85, 389.7