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Coexpression of the Lysyl Oxidase-Like Gene (LOXL) and the Gene Encoding Type III Procollagen in Induced Liver Fibrosis

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Abstract We have isolated a mouse lysyl oxidase-like (LOXL) cDNA from a mouse embryo cDNA library and used this cDNA to measure changes in steady state levels of LOXL mRNA during the development of carbon tetrachloride-induced liver fibrosis in adult mice. These results revealed the coincident appearance of increased steady state levels of LOXL mRNA and type III procollagen mRNA early in the development of liver fibrosis. In contrast, steady state levels of lysyl oxidase mRNA increased throughout the onset of hepatic fibrosis and appeared in parallel with the increased steady state levels of pro-αI (I) collagen mRNA. These findings suggest that the LOXL protein (possibly an isoform of lysyl oxidase) is involved in the development of lysine-derived cross-links in collagenous substrates. Moreover, the substrate specificity of the LOXL protein may be different to that of lysyl oxidase and this difference may be collagen-type specific. J. Cell. Biochem. 72:181–188, 1999.

Key words: LOXL; liver fibrosis; mouse; immunohistochemistry

Lysyl oxidase (LOX) is a copper-dependent enzyme responsible for the development of lysine-derived cross-links in structural extracellular matrix proteins in most tissues, polymerizing collagen and elastin into insoluble structural fibers [Kagan, 1986; Kagan and Trackman, 1991]. This critical cross-linking role of lysyl oxidase in normal tissue development is evident from the multiple abnormalities that arise in both genetic and acquired diseases of connective tissue following changes in lysyl oxidase activity. Increased lysyl oxidase activity has been found associated with lung fibrotic diseases, atherosclerosis [Kagan, 1986] and rat hepatic fibrosis [Wakasaki and Ooshima, 1990]. A decrease in the enzyme activity is associated with Menkes' syndrome, type IX Ehlers-Danlos syndrome [Kuivaniemi et al., 1986; Byers 1995] and congenital cutis laxa [Khakoo et al., 1997]. Both decreased and elevated levels of lysyl oxidase activity were observed in Wilson disease patients [Kemppainen et al., 1997]. Lysyl oxidase has also been shown to act as a tumor suppressor [Contente et al., 1990; Kenyon et al., 1991; Hajnal et al., 1993], indicating that the enzyme is important not only in maintaining the integrity of extracellular matrix structure but also in regulating cell proliferation.

While the expression of lysyl oxidase has been well characterized, the specific mechanisms by which this enzyme interacts with a number of different substrates including elastin and various collagen types is currently unknown. One possibility is that multiple functional variants of lysyl oxidase exist, which may have different specificities toward different types of substrates. Several chromatographic variants of lysyl oxidase were isolated from different tissues of several species including chicken, bovine and human [Kagan et al., 1979; Kuivaniemi et al, 1984; Stassen, 1976]. The mechanistic and functional basis for these variants of lysyl oxidase is unknown. It is clear, however, that these variants of lysyl oxidase

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individually catalyze cross-link formation using both collagen and elastin substrates [Williams and Kagan, 1985]. Moreover, these isoforms are not proteolytically-processed precursors of one another nor do they seem to be posttranslationally modified variants of a secreted precursor protein [Sullivan and Kagan, 1982; Kuivaniemi, 1984; Williams and Kagan, 1985], suggesting that the isoforms may be derived from different genes. In support of this hypothesis, a novel cDNA that encodes a protein with extensive sequence homology to the mature, secreted form of human lysyl oxidase has been recently identified [Kenyon et al., 1993; Kim et al., 1995]. Alignment of the derived amino acid sequences of the lysyl oxidase-like protein and lysyl oxidase revealed conservation of several functional domains such as a copper binding domain, two putative metal binding domains and a growth factor receptor domain in the predicted amino acid sequence of the lysyl oxidase-like cDNA [Kim et al., 1995], implying that the two proteins are functionally related. It has, therefore, been suggested that the lysyl oxidase-like gene may encode an isoform of lysyl oxidase [Kim et al., 1995].

To test the hypothesis that LOX and LOXL may share similar but distinct functions in the synthesis and assembly of connective tissue proteins, we have used a well established model of induced liver fibrosis, in which previous work had noted a distinctly temporal appearance of different collagen types following treatment of rats with carbon tetrachloride [Pierce et al., 1987]. The results we have obtained with this approach have clearly indicated that changes in LOXL gene expression are associated with early changes in liver fibrosis particularly with changes in the synthesis of type III collagen.

MATERIALS AND METHODS

Carbon Tetrachloride Treatment

Experimental adult male mice received twice-weekly intraperitoneal injections of 25% (vol./vol.) carbon tetrachloride in mineral oil at doses of 2 ml/kg of body weight. Groups of four to six mice, each receiving one to eight injections were sacrificed 3 days after the last injection. Control groups of mice that did not receive any injections were also sacrificed with mouse groups that received one, three, six, and eight injections.

Tissue Samples

Body and whole liver weights were recorded at autopsy. The middle lobe of the liver was removed and a specimen was taken for histology. Histological specimens were fixed in Carson's buffered formalin, mounted, and stained with hematoxylin and eosin or Masson's trichrome or used for immunohistochemistry. The remaining tissue from the middle lobe was diced finely with a scalpel and stored at −70°C for RNA extraction.

Quantitative Analysis of mRNA Levels

Total RNA was isolated from homogenized liver tissue samples using RNAzol B solution (Tel-Test Inc., Friendswood, TX). For quantitative analysis a 150 ul mixture of each RNA sample containing 10 ug of liver RNA was prepared on ice. This mixture contained 75 ul of 10 × SSC-formaldehyde (1:1), liver RNA and water to bring the volume to 150 ul. The samples were heated at 65°C for 15 min, placed on ice for 10 min, and immediately applied to a precut nitrocellulose filter (Schleicher and Schuell, Inc., Keene, NH) under vacuum using Minifold slot blot apparatus. Each slot was rinsed three times with 200 ul of 10 × SSC. After blotting, the filters were air-dried and baked at 80°C under vacuum for 2 h. The filters were prehybridized and hybridized with a ^{32}P-labeled cDNA probe in 50% formamide, 5 × SSPE, 10 × Denhard's solution, 2% SDS, and 100 ug/ml denatured salmon sperm DNA at 42°C for 16 h, washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.1 × SSC, 0.1% SDS at 42°C. The amount of filter-bound radiolabeled material was quantitated by phosphor imaging using a Molecular Dynamics model 425E phosphoimager (Sunnyvale, CA) and computer software.

cDNA Probes

Procollagen type I (αI) cDNA probe SP6-667, procollagen type III [Pierce et al., 1987], tropoelastin cDNA probe REL-124d [Pierce et al., 1992], lysyl oxidase cDNA, HLO-2 [Mariani et al., 1992] and a lysyl oxidase-like cDNA probe LOXL-1 [Kim et al., 1995] were used for slot blot hybridizations. A random primer labeling kit (USB, Cleveland, OH) was used to prepare DNA probes and the labeled DNA was purified from unincorporated radioisotopes on a Sephadex G-50 column.

Northern Blot Analysis

Five ug aliquots of total RNA extracted from control and carbon tetrachloride-treated mice liver samples were size separated on 1% formaldehyde-agarose gel and transferred to Hybond-N Nylon membrane (Amersham, Arlington Heights, IL). The RNA was UV cross-linked to the filter, prehybridized, and hybridized in 0.125 M $NaPO_4$, pH 6.8, 0.25 M NaCl, 7% SDS, 1 mM EDTA pH 8.0, 10% PEG 6,000/8,000 and 50% formamide to ^{32}P labeled mouse LOXL cDNA probe overnight. The filter was washed in 1 × SSC, 0.4% SDS at 55°C, and exposed to X-ray film.

Screening of a Mouse Embryo cDNA Library

Phage plaques from a mouse embryo cDNA library (Clontech, Palo Alto, CA) were transferred onto Millipore filters (Bedford, MA). The filters were denatured in 1.5 M NaCl, 0.5 M NaOH for 2 min, neutralized in 3 M NaCl, 0.5 M Tris pH 7.4 for 5 min, and UV cross-linked. The filters were then hybridized to a 1.5 kb human LOXL cDNA probe [Kim el al., 1996] radiolabelled with a Megaprime DNA labeling system (Amersham). Hybridization was in Hybrisol^{tm} Solution without formamide (Oncor, Inc., Gaithersburg, MD) overnight at 42°C. Filters were washed three times in 1 × SSC, 0.4% SDS for 20 min at 42°C. Positive plaques were purified and inserts from 100 ng pure phage DNA samples were PCR amplified using primers supplied by Clontech in a 50 ul reaction volume with 2 mM $MgCl_2$. The PCR conditions were the following: 94°C for 1 min, 30 cycles at 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, and 68°C for 3 min. PCR amplified inserts were sequenced from both ends using vector specific primers and Sequenase Version 2 (USB) and sequencing was completed using specific internal primers.

Immunohistochemistry

Immunoperoxidase staining using a streptavidin biotin complex and diaminobenzidine as chromogen was performed on paraffin embedded 5 um tissue sections. The specific lysyl oxidase antibody was raised against a protein encoded by a partial cDNA, a product of the LOX gene and did not give any cross-reaction with the protein encoded by the LOXL gene. A detailed characterization of these antibodies was described earlier [Decitre et al., 1998]. The mouse collagen type I and type III antibodies were kindly provided by the Institut Pasteur de Lyon (reference #20151 and 20351).

RESULTS

The Mouse LOXL cDNA Sequence

Screening of a mouse embryo cDNA library using a 1.5 kb human LOXL cDNA clone resulted in the isolation of two overlapping recombinants with insert sizes of 0.3 kb and a 1 kb. DNA sequence analysis of these inserts confirmed homology with sequences extending from the 3'end of exon 2 to the 5'end of exon 7 of the human LOXL gene. This mouse LOXL cDNA sequence was 88% homologous to the corresponding domain of the human LOXL cDNA sequence and 73% homologous to the mouse LOX cDNA (Fig. 1).

Northern Analysis

Total liver RNA obtained from mice exposed to three injections of carbon tetrachloride (Fig. 2, lane 1) and untreated mice (Fig. 2, lane 2) was hybridized to the 1 kb mouse LOXL cDNA probe. A single mRNA species of 2.4 kb was detected which is identical in size to the human lysyl oxidase-like mRNA.

Carbon Tetrachloride-Induced Liver Fibrosis

The chronic administration of carbon tetrachloride to adult mice resulted in the progressive development of necrosis, cirrhosis and fibrosis in the livers of all experimental animals. Visual examination of the livers revealed fatty foci following a single acute injection of carbon tetrachloride. After three injections, extensive thickening of the liver edge, adhesions, and fibrosis were observed. Histopathologic evaluation of liver samples following the first injection showed large fields of hepatocyte necrosis localized in the medio-lobular and centro-lobular areas (Fig. 3A). Following three injections an inflammatory infiltrate developed in the portal areas and Kupffer cells were hyperplastic in the lobular parenchyma (Fig. 3B). Collagen deposition was observed in the thin connective tissue bordering the portal spaces and extended from the portal spaces into the lobule (Fig. 3C). Following eight injections, the liver samples showed persistent inflammatory infiltration and septal connective bridging across the lobule from portal space to the centro-lobular vein; collagen deposition was now clearly present in these intra-lobular connective bridges (Fig. 3D).

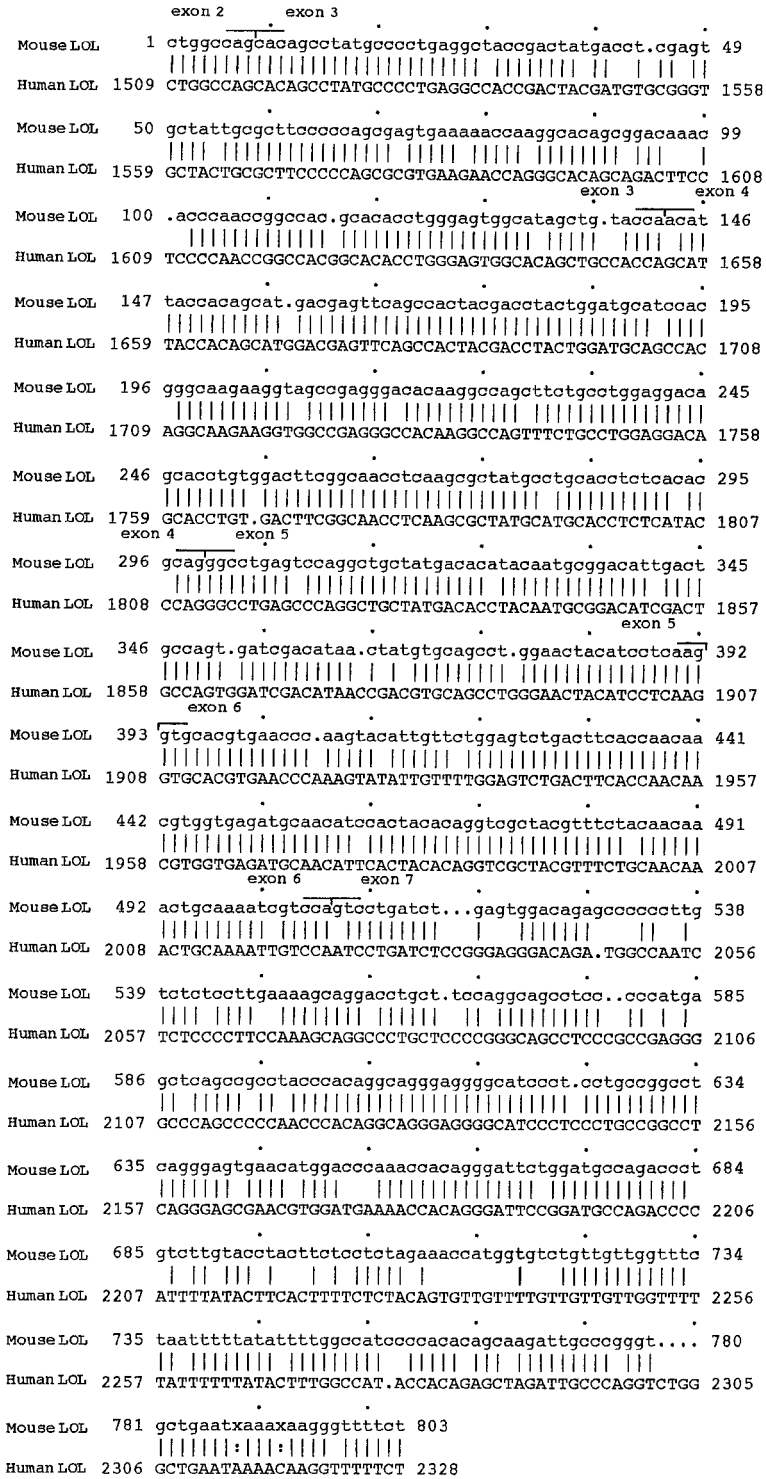

Fig. 1. The mouse lysyl oxidase-like cDNA sequence. The mouse cDNA sequence is compared to the corresponding region of the human lysyl oxidase-like cDNA sequence. Exon-intron boundaries as they appear in the human gene are marked. Spaces (.) in the sequence permit the alignment of insertions.

Localization of the LOXL Protein to the Extracellular Matrix

Immunohistochemistry was performed with lysyl oxidase, collagen type I, collagen type III, and lysyl oxidase-like antibodies using fibrotic liver sections obtained after three injections of carbon tetrachloride. Collagen type I and lysyl oxidase immunostaining (data not shown) and collagen type III (Fig. 3E) immunostaining was observed in the portal spaces and extended into the lobule. Staining with the lysyl oxidase-like antibody was similarly detected around the por-

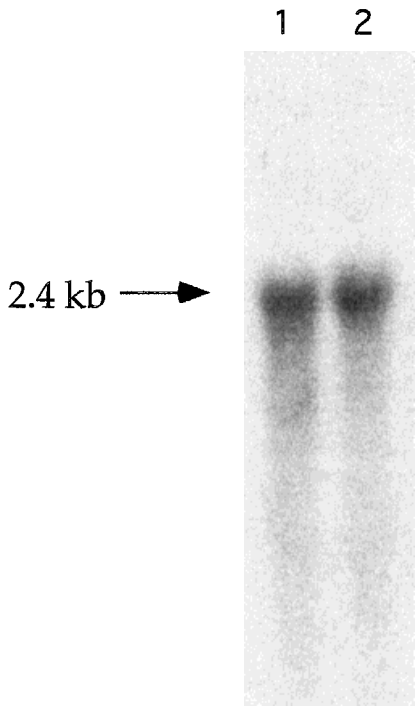

Fig. 2. Northern blot analysis of the mouse lysyl oxidase-like transcript. Total RNA, extracted from a mouse liver sample treated with three injections of carbon tetrachloride (**lane** 1) and from an untreated control (**lane 2**) was hybridized to a 1 kb radiolabelled mouse LOXL cDNA. The size of the single species of mRNA detected is indicated with an arrow on the left of the panel.

tal spaces, extended into the lobule and localized the LOXL protein into the extracellular matrix to sites of active fibrosis (Fig. 3F). In normal liver sections of control animals of the same age there was no LOXL protein detected. Small amount of collagen type III was localized to the portal spaces as thin reticular sheets surrounding the portal triad radices.

Quantitative Analysis of mRNA Levels

We followed the course of carbon tetrachloride-induced liver fibrosis from a single injection to eight injections of carbon tetrachloride over a period of 4 weeks. Steady state levels of mRNAs encoding lysyl oxidase, LOXL, elastin, pro-α I (type I) collagen and pro-α I (type III) collagen were measured and the results are summarized in Figures 4 and 5. A moderate but statistically significant increase of lysyl oxidase mRNA was observed over the 4-week period of carbon tetrachloride-induced liver fibrosis with an approximately two-fold increase following eight injections of carbon tetrachloride.

A significant increase of the pro-α I (I) collagen mRNA was observed after six injections of carbon tetrachloride. The almost four-fold increase in pro-α I (I) collagen mRNA levels coincided with the extensive deposition of total collagen in the liver section as observed by Masson's trichrome staining. Tropoelastin mRNA levels did not change significantly over the experimental period (Fig. 4), in agreement with a number of previous reports showing that liver fibrosis is mainly due to deposit of collagen [Pierce et al., 1987; Hayasaka et al., 1991; Ala-Kokko et al., 1992; Brenner et al., 1993, Bedossa et al., 1994]. Levels of pro-α I (III) collagen mRNA were observed to increase early in the development of liver fibrosis and reached a maximum following only three injections of carbon tetrachloride. Steady state levels of lysyl oxidase-like mRNA followed a very similar profile, reaching a 2.6-fold increase in mRNA levels after three injections of carbon tetrachloride (Fig. 5).

DISCUSSION

Chronic administration of carbon tetrachloride has been known to induce fibrosis and cirrhosis in the liver and is characterized by increased deposition of interstitial collagen in the extracellular matrix [Rubin et al., 1963; Unakar, 1966; Hayasaka et al., 1991]. In normal liver, collagen is found only in small amounts (approximately 4%), whereas significant increases of procollagen type I, III, and IV were observed in carbon tetrachloride-induced hepatic fibrosis and cirrhosis. Changes in levels of mRNA encoding different collagen types do not occur simultaneously. It was previously shown that the level of type III procollagen mRNA increased significantly at an earlier stage of carbon tetrachloride-induced fibrosis while the mRNA levels of types I and IV procollagens increased at later stages [Pierce et al., 1987]. This separate temporal expression of different types of procollagens has also been observed in Schistosoma mansoni-induced hepatic fibrosis of mice; the deposition of extracellular matrix proteins starts mainly with type III collagen around the eggs in granulomas, followed by a peak of type I collagen production later in the development of fibrosis [Grimaud et al., 1987]. In agreement with these findings, we have observed separate increases of type I and type III procollagen mRNA during the development of carbon tetrachloride-induced liver fibrosis.

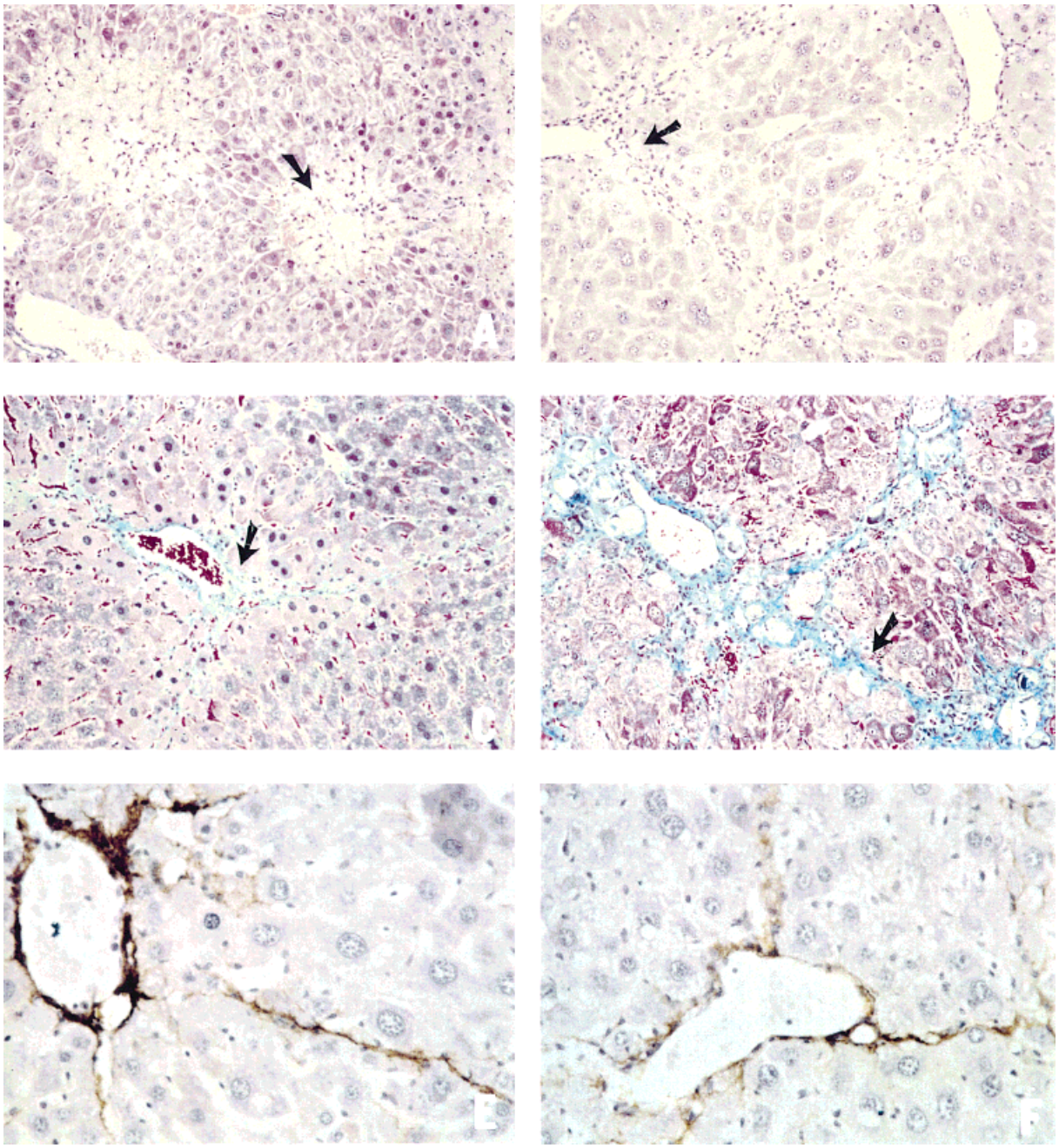

Fig. 3. Histological staining of fibrotic mouse liver and immuno-localization of the lysyl oxidase-like protein. Hematoxylin-eosin staining of liver sections following a single injection (**A**) and three injections (**B**) of carbon tetrachloride. Masson's trichrome stain of liver sections following three (**C**) and six (**D**) injections of carbon tetrachloride. Immunohistochemical staining of liver sections following three injections of carbon tetrachloride with a lysyl oxidase-like antibody (**E**) and a collagen type III antibody (**F**). Magnification: 16(A,B,C,D) 20(E,F).

An increase in lysyl oxidase activity has previously been observed in carbon tetrachloride-induced hepatic fibrosis and cirrhosis [Carter et al., 1982] and in murine schistosomiasis [Sommer at al., 1993]. Lysyl oxidase activity was increased four-fold in the liver of carbon tetrachloride-treated rats and was completely inhibited by BAPN, a specific inhibitor of lysyl oxidase [Wakasaki and Ooshima, 1990]. In confirmation of these earlier findings, we have shown a significant increase in the steady state levels of LOX mRNA during the development of liver fibrosis in our mouse model of this disorder. This increase in LOX mRNA levels coincided

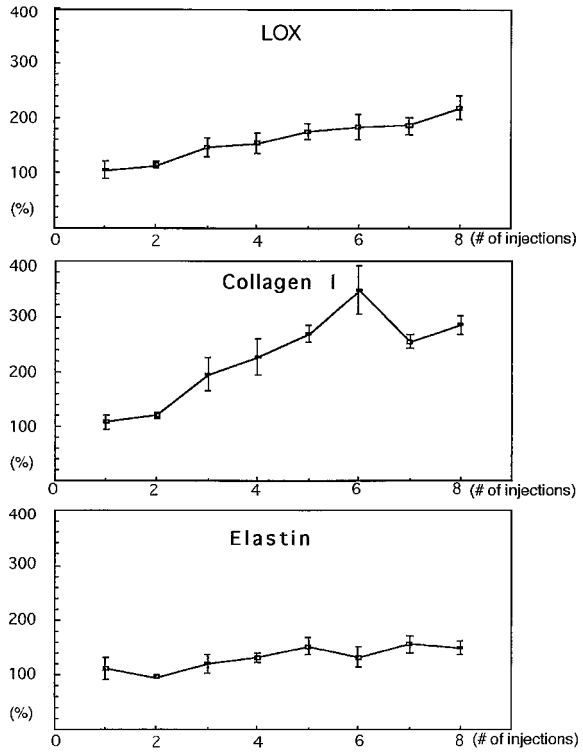

Fig. 4. Steady state mRNA levels of lysyl oxidase, procollagen α1(I), and elastin during carbon tetrachloride-induced liver fibrosis. The results are expressed as a percent of the mean control of each mRNA from uninjected mice (n = 6). The data are reported as mean ± standard error. The mean control is set to 100% and the standard error expressed as a percent of the mean. Each vertical bar indicates a standard error at each injection. *P*-value at the highest peak of each mRNA were calculated using a students *t*-test. *P* (lysyl oxidase) < 0.02 *P* (procollagen αI(I) < 0.01 *P* (elastin) > 0.1 (not statistically significant).

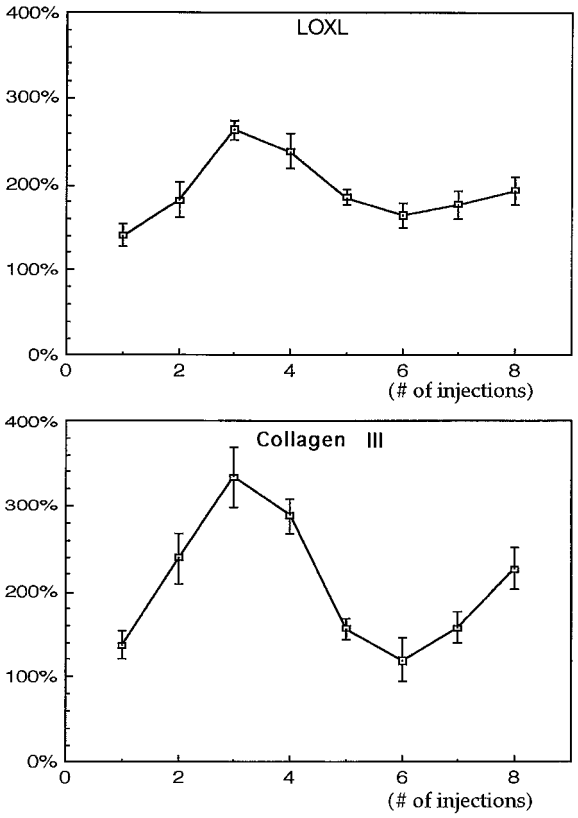

Fig. 5. The mRNA levels for pro αI (III) collagen and the lysyl oxidase like protein (LOXL) during carbon tetrachloride-induced liver fibrosis. The results are expressed as a percent of the mean control of each mRNA from uninjected mice (n = 6). The data are reported as mean ± standard error. The mean control is set to 100%, and the standard error expressed as a percent of the mean. Each vertical bar indicates a standard error at each injection *P* (LOXL) < 0.001 and *P* (collagen III) < 0.01.

with an increase in the levels of pro αI (I) collagen mRNA and contrasted strikingly with the concomitant increase in mRNAs for both LOXL and pro αI (III) collagen. Parallel biosynthetic changes in LOX and pro αI (I) collagen suggests that an increased synthesis of this form of lysyl oxidase occurs in response to increased synthesis of type I collagen, possibly as this variant of lysyl oxidase is responsible for the development of lysine derived cross-links in type I collagen during the development of hepatic fibrosis. Similarly, the parallel changes in mRNA levels for pro αI (III) collagen and LOXL suggests that this homologue of lysyl oxidase is synthesized early in the development of liver fibrosis, in response to the increased synthesis of pro α I (III) collagen and is possibly responsible for the development of cross-linked type III collagen.

Several authors have previously indicated that it is not clear how a single lysyl oxidase can catalyze the oxidative deamination of a diverse range of substrates [Eyre et al., 1984]. The extensive homology between LOX and LOXL suggests a functional similarity between these proteins. While it is well known that LOX is located in regions of active fibrosis, the results we have presented in this manuscript, using an antibody to LOXL, indicate colocalization of LOXL to the extracellular matrix and in this model of liver fibrosis, to regions active in collagen synthesis and deposition. Moreover, in an in vitro assay of cross-link activity of isolated LOXL we have demonstrated significant lysyl oxidase activity [Decitre et al., 1998]. Taken together, this data strongly suggests that LOXL is indeed a variant of lysyl oxidase. The existence of such genetically distinct variants of this enzyme might be one mechanism to explain how lysyl oxidase may function on multiple substrates. In support of this hypothesis,

the results presented in this manuscript suggest that the cross-linking of type III collagen fibrils is mediated by the oxidative deamination of lysine residues catalyzed by newly synthesized LOXL. Further, the lysine-derived crosslinks formed in type I collagen are formed through the action of a variant of this enzyme that is encoded by the LOX gene and is referred to in this work as lysyl oxidase.

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