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Premalignant Variations in Extracellular Matrix Composition in Chemically Induced Hepatocellular Carcinoma in Rats

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Abstract Chemical composition of extracellular matrix (ECM) plays a pivotal role in cellular and tissue development, regeneration, and differentiation. It also plays a key role in pathogenesis of hepatocellular carcinoma (HCC). This study explored premalignant changes in the liver tissue content of collagen (as hydroxyproline, HP), total glycosaminoglycans (TGAGs), free glucosamine (FGA), total sialic acid (TSA), lysosomal membrane integrity variations (calculated as total and free cathepsin D activities), and liver histology. Serum alfa-fetoprotein (AFP) level was used as an early marker for HCC in two groups of Wistar rats. One group of rats served as control and was provided normal saline orally. The other group was provided trichloroacetic acid (TCA) as 0.5 g/kg/day for five consecutive days by oral gavage. Animals were killed before tumor development. The treatment revealed dysplastic changes in addition to microsteatosis (fatty changes). Both sinusoids and the portal vein among dysplastic cells were dilated and congested. These dysplastic foci are believed to be premalignant and may be precancerous lesions. The following things were observed: a highly significant increase in serum AFP (as a key marker for HCC), a significant decrease in HP and TSA, a significant increase in FGA, nonsignificant decrease in TGAGs, significant up-regulation of free cathepsin D, nonsignificant decrease in total cathepsin D activities, and destabilization of lysosomal membrane integrity. Down-regulation of HP, TSA, and TGAGs seems to be a prerequisite for cancer development. This might be stimulated by up-regulation of free cathepsin D activity. Perhaps tissue fibrosis is not a condition for developing HCC because collagen was significantly depressed. Up-regulated FGA could be assumed to be a defense mechanism against TCA-induced proteolysis of membrane proteins because it is frequently reported to be of value in cancer chemotherapy. Studied ECM perturbations can be assumed as preliminary changes during chemical hepatocarcinogenesis at the tissue level. Prospective studies on blood levels of cathepsins, TGAGs, and individual ECM variables such as TSA, FGA, and Hp in patients at risk for HCC, performed in parallel with assessments of AFP, may provide a cost-effective way to find new links between tissue changes and circulation that would permit early prediction of disease. It may also provide a way to monitor HCC and compensate for the missed peak AFP values.

Keywords Premalignancy · Hepatocellular carcinoma · Collagen · Total glycosaminoglycan · Glucosamine · Sialic acid · Cathepsin D · Alfa-fetoprotein · Diagnosis · Dysplasia · Rats

Extracellular matrix (ECM) is a complex macromolecular structural network, surrounding stromal cells and underlying the majority of endothelial and epithelial cells. It modulates hepatic development and regeneration, and it maintains normal tissue architecture and differentiated state (Martinez-Hernandez and Amenta 1995). Both glycosaminoglycans (GAGs) and collagen, along with structural proteins, constitute the key components for the cell surface–ECM interface. Collagen provides a necessary scaffold for cellular attachment and tissue formation, while complex polysaccharides (GAGs) fill the scaffold. This complex nature can

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control cell signaling and growth in response to cytokines and growth factors (Esko and Lindahl 2001; Sasisekharan and Venkataraman 2000). Globally, ECM plays a decisive role in the pathogenesis and progression of hepatocellular carcinoma (HCC) by disturbing cellular proliferation, differentiation, and apoptosis, favoring invasion and metastasis (Wu et al. 2006). It has been proposed that degradation of tumor cell-surface GAGs represents a switch from a primary tumor to a metastatic disease state (Hulett et al. 1999; Vlodavsky et al. 1999). GAGs include chitin, hyaluronic acid, keratin sulfate, and glucosamine (2-amino-2-deoxy-alpha-D-glucose), which is an amino saccharide found in almost all tissues, but with its highest concentration in liver, kidney, and cartilage. In addition, glucosamine is a fundamental building block in the synthesis of glycolipids, glycoproteins, GAGs, and proteoglycans (de los Reyes et al. 2000). Sialic acid (SA, *N*-acetyl neuraminic acid) has been found to be a constituent of the plasma membrane of mammalian cells. Through *O*-glycosidic linkage, SA binds to *N*-acetyl galactosamine, which in turn binds to protein either directly or via other carbohydrate moiety, forming glycoproteins (Langley and Ambrose 1967). Early investigations showed that SA content is disturbed in hepatoma membranes than normal liver cells (Benedetti and Emmelot 1967). Enzymatic degradation of ECM components was reported to be an underlying cause of cancer cell growth, proliferation, and migration (metastasis) (Goll 1989; Ratnikov et al. 2000).

The metabolism of functional proteins is a continuous process involving proteolysis, which is the destruction of individual protein molecules and their replacement through protein synthesis. Intracellular proteolysis includes either lysosomal (cathepsins) or nonlysosomal pathway, which is mostly executed by calpains and proteasomes (Johari et al. 1993; Millward et al. 1981; Thompson and Palmer 1998). The rate of degradation of both intracellular and extracellular proteins is greatly dependent on cathepsin activity. Thus, the degradation rate of proteins can be monitored by measuring cathepsin activity oscillations (Gebbia et al. 1985). Lysosomes are membrane-bound structures containing hydrolytic enzymes capable of degrading most of the cellular constituents. They also play a pivotal role in secretion and transport processes. Leakage of lysosomal enzymes accounts for many tissue derangements and target organopathies (Murray et al. 1999; Subashini et al. 2007). They are found in all animal cells and are more numerous in disease-fighting cells. Lysosomal enzyme disorders contribute to several human diseases, either as a result of genetic defects in its enzyme expression or as a result of the escape of lysosomal enzymes (lysozymes) into an extralysosomal microenvironment (Ng et al. 1992). HCC is one of the cancers with the worst prognosis in the world. It develops mostly over chronic liver diseases of cirrhotic fate. HCC nodules are always encapsulated by excessive ECM materials among a bed of cirrhotic tissue (Yates and Kensler 2007). It causes nearly 600,000 deaths each year (Stetler-Stevenson et al. 1993).

ECM degradation by specific proteases was reported to play an important role in cancer invasion and metastasis (Parnell et al. 1986). Serum alfa-fetoprotein (AFP) level is still the gold standard in laboratory assessment of HCC worldwide (Acharya et al. 1995, 1997; Goldsworthy and Popp 1987; Kroes et al. 1973; Mather et al. 1990; Parnell et al. 1988).

Trichloroacetic acid (TCA) is frequently used as a preemergence herbicide, medically as a caustic agent for chemical cautery, and as a common laboratory agent. These direct uses of TCA are considered minor sources of environmental contamination (Page 1981). It is metabolically related to trichloroethylene, an organic solvent with diverse applications that is a contaminant of surface water and groundwater (Love 1982; Mink et al. 1983). It has previously been demonstrated that oral administration of sodium hypochlorite induced enteric production of both di- and trichloroacetic acids (El Combe 1982). In addition, oral administration of TCA induced hepatic peroxisomal proliferation in mice and rats (El Combe 1982, 1985). In a recently published work, De Angelo et al. (2008) reported that administration of TCA in drinking water resulted in hepatocellular neoplasia in mice. The mode of carcinogenic action of this chemical was considered to be the result of increased cellular proliferation, lipoperoxidation, and liver weight.

This study was conducted to pursue early changes in ECM per TCA preneoplastic challenge, with serum AFP level taken as a classical indicator for chemically induced HCC. The changes in collagen (represented as hydroxyproline [HP], which constitutes approximately 14% of tissue collagen), total GAGs, free glucosamine (FGA), total sialic acid (TSA), and tissue proteolytic enzyme activity, shown by cathepsin D were estimated. The activity ratio of total to free cathepsin D was considered as an index for lysosomal membrane integrity. This study sought to assess some early variations in ECM components thought to be released into general circulation in a trial to pursue premalignant changes; to study this early variations, we killed the experimental animals before they developed HCC. Our findings may be of future value in the early prediction of HCC.

Materials and Methods

Materials

Animals

Sixteen male Wistar rats weighing a mean ± standard error of 190 ± 10 g were used throughout this study. The

animals were kept in polyethylene cages in a moderately humid room under a 12-h light/12-h dark cycle. They were provided an ordinary rodent diet and water ad libitum, and left to acclimate for a week before the experiment began. They were divided into two groups of eight rats each.

The first group was given a single daily oral dose of saline at the same volume of the treated group for five consecutive days. The second group of animals was given single oral doses of TCA as 0.5 g/kg body weight for five consecutive days. The pH of TCA solution was adjusted to 6.5–7.5 by using 0.1 N NaOH solution before administration (Tao et al. 2000).

Twenty-four hours after the last dose, blood samples were withdrawn from the neck vein. The blood was centrifuged to separate sera, frozen directly in liquid nitrogen, and kept at −80°C until use in the analytical procedures. Livers were excised, washed with saline, and divided into two parts; one was directly frozen, and the other was kept in 10% formalin solution for histological study.

Chemicals

All chemicals were purchased from local agencies and were of high purity and analytical quality.

Methods

Determination of rat serum AFP levels was performed by enzyme-linked immunosorbent assay kits (UBI MAGIWEL AFP quantitative CM-101) following the manufacturer's instructions (Belanger et al. 1973).

Determination of HP as a Representative for Liver Tissue Collagen

HP was primarily extracted from tissues by acidic hydrolysis. Briefly, 250 mg was thoroughly homogenized with 5 ml 6 N HCl, subjected to heating in a water bath for 3 h at 130°C in sealed tubes, and cooled. The volume was returned to 5 ml by 6 N HCl to compensate for losses due to evaporation, and the pH was adjusted to 6–7 by adding 0.1 N NaOH solution (Woessner 1961). HP content was estimated in the hydrolysate by adding to 1 ml, 1 ml of 0.01 M $CuSO_4$ solution, 1 ml 2.5 N NaOH, and 1 ml 6% H_2O_2. The mixture was shaken vigorously, then heated at 80°C for 5 min with shaking, and cooled by chilling. Four milliliters of 3 N H_2SO_4 was added, followed by 2 ml 5% ρ-dimethylaminobenzaldehyde solution in *n*-propanol kept in water bath (70°C) for 16 min; the solution was cooled in tap water. The color that was produced was read at 540 nm against standard HP (Sigma) (Neuman and Logan 1950).

Determination of TGAGs

A given liver tissue weight was extracted by five volumes of 4 M guanidinium HCl (from UFC) in 0.05 M sodium acetate, containing both 0.01 M di-sodium EDTA and 0.005 M benzamidine HCl (from Merck) (both used as protease inhibitor), leaving the mixture for 5 h at 4°C until extraction occurred while cold. After centrifugation, the supernatant was kept for colorimetric investigation (Oegema et al. 1975). TGAGs was estimated in the hydrolysate by adding to 0.1 ml of hydrolysate, 1.2 ml Alcian blue 8GX (from BDH), prepared as 1.4 mg/ml of 0.5 M sodium acetate. Absorbance was read at 480 nm against standard heparin sodium (from Nile Co.) (Gold 1979).

Determination of FGA

Before extraction, the tissue was defatted by chloroform/methanol (2/1 v/v) and centrifuged, and the supernatant was discarded (Folch et al. 1957). The defatted tissue was then hydrolyzed with 0.1 N H_2SO_4 at 80°C for 1 h and centrifuged. The clear supernatant was used for FGA determination after addition of an equal volume of 0.1 N NaOH (Elson and Morgan 1933). Briefly, 1 ml of neutralized hydrolysate was added to 1 ml of acetyl acetone solution in 0.5 N sodium carbonate (as 1 ml/50 ml), and 1 ml of water was added. Tubes were heated in a boiling-water bath for 15 min and cooled. Ethanol was added to compensate for evaporation and to complete the volume to 3 ml. A total of 1 ml of Ehrlich reagent was added. Ehrlich reagent is composed of 0.8 g ρ-dimethylaminobenzaldehyde dissolved in 30 ml ethanol plus 30 ml concentrated HCl. Ethanol was added to complete the reaction mixture volume to 10 ml. The red color that was produced was read at 540 nm after 20 min against standard glucosamine HCl (BDH) (Pari 2006).

Determination of TSA

The previously defatted tissue hydrolysate in 0.1 N H_2SO_4 was directly used for estimation of TSA content according to the method of Warren (1959). In brief, 0.2 ml of hydrolysate was added to 0.1 ml of 0.2 M sodium periodate (meta) in 9 M phosphoric acid. The tubes were shaken and left to stand for 20 min at room temperature. A total of 1 ml 10% sodium arsenite (in 0.5 M Na_2SO_4 + 0.1 N H_2SO_4) was mixed well with the mixture until the yellowish-brown color disappeared. Then 3 ml of thiobarbituric acid solution (0.6% in 0.5 M Na_2SO_4) was added. The tubes were sealed and heated for 15 min in a vigorously boiling water bath. After cooling, 1 ml of the mixture was transferred to a tube containing 1 ml cyclohexanone,

shaken, and centrifuged for 3 min. The absorbance of produced red color in the upper organic phase was read at 549 nm against standard SA (Sigma).

Determination of Lysosomal Membrane Integrity

Extraction of the target enzymes from liver tissue was performed by homogenizing 300 mg of liver tissue with 6 ml of ice-cold distilled water, as described elsewhere (Kumar 2000). Total protein was determined in the enzyme substrate before enzyme assessment was performed following the method of Lowry et al. (1951); the total protein value was 670 mg/dl.

Estimation of Lysosomal Membrane Integrity

The estimation of the lysosomal membrane integrity variable relied on determination of cathepsin D proteolytic activity of the tissue extract on a substrate containing 1% egg albumin as the substrate protein for enzyme actions (Anson 1938). Colorimetric estimation of tyrosine as the product of proteolysis was conducted by diluted Folin–Ciocalteu reagent (Folin 1927) against a tyrosine standard (Acharya and Katyare 2004; Kumar et al. 2000) after acidifying the protein substrate (for determining free cathepsin D activity). Next, dilution of the extract and frequent cycles of freezing and thawing were applied to determine total cathepsin D activity (Acharya et al. 2004; Barret 1977; Khandkar et al. 1996; Nerurkar et al. 1988). Enzyme activity in all cases was considered as micrograms of released tyrosine per milligram of substrate protein per deciliter. Total/free cathepsin D activity was considered as a measure of lysosomal membrane integrity (Khandkar et al. 1996; Nerurkar et al. 1988).

Statistical Analysis

The presented data were expressed as mean ± standard error (SE). Statistical significance was examined by one-way analysis of variance (Duncan 1955). *P* values less than 0.05 were considered to be statistically significant.

Results

Biochemical Results

Administration of TCA significantly up-regulated serum AFP level, depressed both liver HP and TSA, but resulted in significantly high FGA tissue content ($P < 0.01$). TCA nonsignificantly down-regulated TGAGs tissue content, significantly up-regulated free cathepsin D, and nonsignificantly affected total cathepsin D enzyme activities, with nonsignificant reduction of lysosomal membrane integrity in treated rats (Tables 1, 2).

Histological Results

Administration of TCA showed hyperchromatic nuclei with more than one nucleolus, steatotic changes, and ballooning of hepatocytes, in addition to vascular congestion. These changes together constitute a premalignant tissue change (Figs. 1, 2, 3 and 4).

Discussion

Cancer arises as a consequence of profound modifications in tissue structure and composition. This malignant event starts at the cellular and subcellular levels through an interference with the normal program of ECM biosynthesis. Three possible pathways contribute to the malignant process: direct production of some matrix macromolecules, indirect production by malignant cells of interfering factors, or direct action of an environmental carcinogen on normal mesenchymal cells (Kadar et al. 2002).

This study assesses the effect of a hepatocarcinogen, TCA, on some ECM components known to contribute to HCC after a short period of administration as an early consequence at the tissue level before reaching the period when HCC develops in order to check the cancer-predisposing ECM variation candidates in the malignant process. The results show that TCA treatment induced dysplastic tissue changes, vascular congestion, hepatocyte ballooning, and microsteatosis; taken together, a premalignant change

Table 1 Variations in serum AFP level (ng/ml) and liver tissue content of HP, TGAGs, FGA, and TSA (μg/g wet tissue) after oral administration of TCA (0.5 g/k/day for 5 days)

Group	AFP	HP	TGAGs	FGA	TSA
Control	7.0 ± 1.63	5.19 ± 0.17	45.5 ± 1.9	58.29 ± 1.23	44.1 ± 1.4
TCA	270.5 ± 17**	1.58 ± 0.1**	44.4 ± 1.27	141.04 ± 6**	8.61 ± 0.64**

Values are expressed as mean ± SE ($n = 8$)

**Significantly different from control at $P < 0.001$

Table 2 Lysosomal membrane integrity index in rat liver tissue after oral administration of TCA (0.5 g/k/day for 5 days)

Characteristic	Control	TCA group
Free cathepsin D activity	5.1 ± 0.11	6.2* ± 0.17
Total cathepsin D activity	3.6 ± 0.13	4 ± 0.05
Lysosomal membrane integrity	0.72 ± 0.03	0.65 ± 0.02

Values are expressed as mean ± SE ($n = 8$)

*Significantly different from control at $P < 0.01$

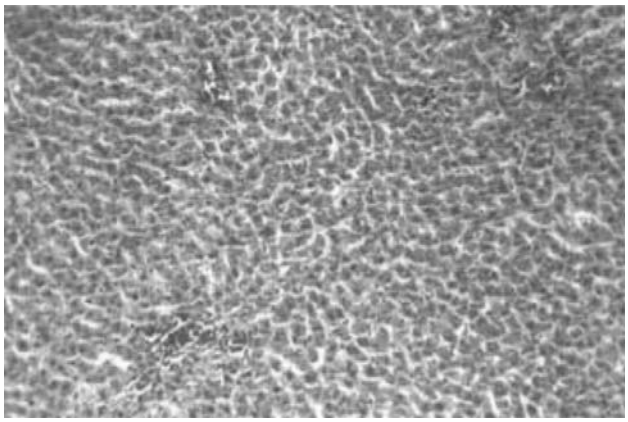

Fig. 1 Control liver tissue. Light microscopical findings of liver tissue (H&E, 300×)

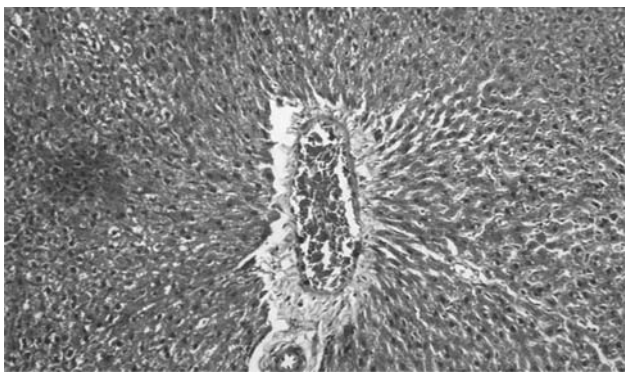

Fig. 2 TCA exerted dysplastic changes (in the lower part of the image), characterized by greater nuclear-to-cytoplasmic ratio, greater nuclear density, and more basophilic cytoplasm. Both sinusoids and the portal vein, among dysplastic cells, were dilated and congested. Light microscopical findings of liver tissue (H&E, 300×)

was seen. A significantly high serum AFP level confirmed that liver cancer began early. Acharya et al. (1995, 1997) reported mild liver histopathological changes after a single low TCA dose in male Wistar rats. Moreover, occasional focal hepatocellular enlargement and intracellular hepatic swelling were noticed in other rat strains exposed to same short-term TCA treatments (Goldsworthy and Popp 1987; Mather et al. 1990; Parnell et al. 1988). In the present

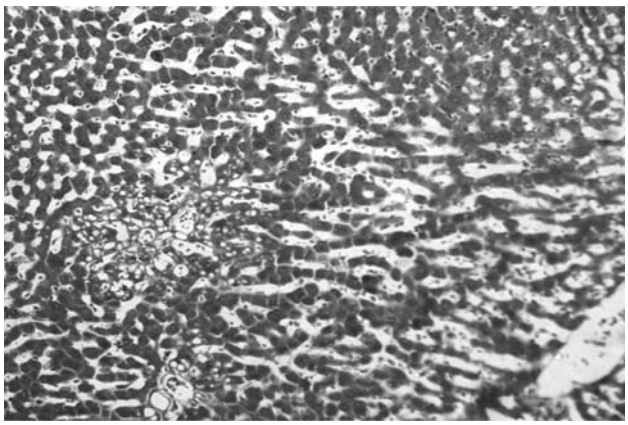

Fig. 3 Another area of dysplasia, which shows microsteatosis (fatty changes). Light microscopical findings of liver tissue (H&E, 300×)

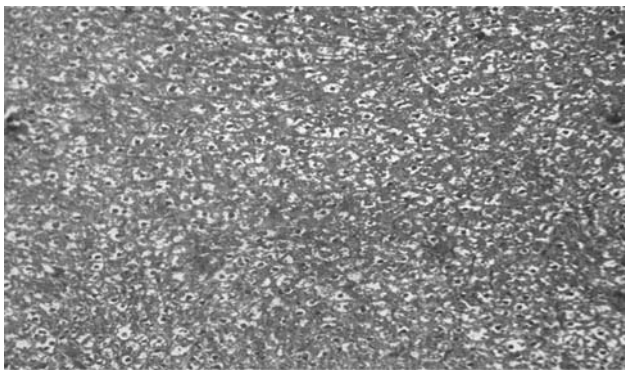

Fig. 4 Marked ballooning of hepatocytes may be observed, as well as dysplastic changes and vascular congestion. These dysplastic foci are believed to be premalignant (precancerous) lesions. Light microscopical findings of liver tissue (H&E, 300×)

study, hepatocytic dysplasia and high AFP levels support the hypothesis of TCA-induced HCC, which seems to be a highly significant acute response. It was reported that AFP values showed a peak against time, indicating a possible misleading in early diagnosis if this peak is missed (Kroes et al. 1973). TCA significantly decreased HP tissue content. This means that collagen synthesis was significantly down-regulated—an effect also observed by Wang et al. (1995), who found that collagen content of liver tissue samples from HCC patients was significantly lower than normal subjects. They referred this action to an increased collagenase expression, which degrades the collagen matrix in HCC nodules and its surrounding liver tissue. Other studies suggested that tumor cells stimulate the expression of matrix metalloproteinases 2 and 9, which contribute to decreased collagen deposition as a response to chemical hepatocarcinogenesis (Taras et al. 2006).

Furthermore, TCA treatment nonsignificantly decreased TGAGs tissue content after this short period. Depletion of both collagen and TGAGs has been suggested to be a major

prerequisite for cellular migration and invasiveness (Kwack et al. 2006). Moreover, enhanced tumor survival and metastasis, and subsequently tumor cell entry into circulation, were reported to be an outcome of cellular detachment after enhancement of matrix glycosaminoglycan degradation (Capurro et al. 2005; Ip et al. 2005). The role of GAG down-regulation in hepatoma growth, migration, and invasion was recently documented in an in vitro study (Sutton et al. 2007). Taken together, a significant TGAGs depletion can be expected by time after onset of initiation. The present work depicted a significant up-regulatory action of TCA on FGA tissue content as an early event before cancer initiation. Generally, hexosamines are important structural components of many membrane glycoproteins and mucopolysaccharides (Fujiwara et al. 1990).

Literature that describes glucosamine (GA) levels in liver cancer is sparse. In the present findings, increased FGA release from the tissue may be referred to its inclusion in fighting proteasome degradation in response to chemical carcinogenesis (Qiu et al. 2005). In other words, FGA up-regulation may be due to enhanced release from the hosting macromolecules after TCA-enhanced proteolytic activity (Werle et al. 2000). The suggestion that FGA may be engaged in cancer control can be supported by the proven antitumor activity of GA and its derivatives in an in vitro investigation (Zhang et al. 2006).

SA is a monosaccharide found typically attached to cell surface glycoconjugates (glycolipids, glycoproteins, and proteoglycans). It plays an important role in many physiologic and pathologic processes, including progression and spread of human malignancies (Varki and Varki 2007). The present results show a significant depletion in liver tissue TSA content. This result is in agreement with the observation of Ganapathy et al. 2008), who found a depletion in SA in many cancer tissues, including liver. It can be assumed that SA tissue depletion is accompanied by a concomitant release into circulation, increasing blood TSA levels in humans (Basoglu et al. 2007; Wongkham et al. 2003) and in animals (Arif et al. 2005).

Lysosomal proteolytic activity in liver cells plays a decisive role in protein metabolism, with cathepsin A and D being the main enzymes involved in catabolic processes (Marzo et al. 2002). Tumor cells require specific proteolytic enzymes for invasion and metastasis, including lysosomal peptidase–cathepsin (Lah et al. 2000). In the present work, TCA treatment depicted nonsignificant up-regulation in total cathepsin D activity, with decrease in lysosomal integrity. However, it significantly activated free cathepsin D activity. Proteolytic enzymes, including endopeptidases, are necessary prognostic indicators after tumor resection; they always increase in patients at risk of recurrence (Schuppan et al. 1998). Possibly disturbed ECM expression in response to hepatocarcinogenesis is a defense mechanism in which these macromolecules bind with low affinity to hepatocyte growth factors sequestering proliferation (Mohammed et al. 2005). However, up-regulated proteolysis, if it persists, may release these complexes, initiating hepatocyte proliferation (Anna et al. 1994).

TCA is one of three metabolites of trichloroethylene, a common solvent known to pollute groundwater (Ferreira-Gonzalez et al. 1995). It is as a member of the peroxisome proliferators, which are responsible for many mutations leading to diverse target organ cancers. The early down-regulatory effect of TCA on lysosomal membrane may contribute to the mechanism of cancer induction and invasion by this substance, now being observed for the first time. It is clear in these results that disruption of lysosomes will lead to increased proteolytic activity that likely initiates neoplastic invasiveness.

It is well established that cancer can activate enzyme leakage into pericellular components (Braun et al. 1978).

It can be concluded that TCA carcinogenesis probably through a proteolytic potential, an effect accompanied by depleted TGAGs, collagen, and TSA, with high FGA contents. These changes together could initiate hepatocarcinoma. Liver dysplasic changes along with significant AFP increases support the assumption of ECM perturbation and emphasize the utility of the selected biomarkers as useful members in a panel strengthening AFP specificity and specificity in HCC diagnosis, taking into account that AFP may decrease sometime after tumor development, while other variables may persist as reliable diagnostic tools. The change in the variables studied necessitates prospective studies on blood to assess a possible escape into circulation. This may contribute to a new trend in early HCC assessment and prognosis. Early changes in lysosomal membrane integrity that are manifested by increased release of proteases, disrupted ECM synthesis, and individual ECM variations together may comprise a new line of research that may help us add more specific and sensitive early diagnostic biomarkers in HCC. The missed-peak AFP value and decreased sensitivity and specificity may be greatly potentiated if the studied parameters were pursued in the blood of candidate cases at risk for HCC. The present study aimed to pursue changes that would help detect disease early, but further studies may be needed to implement this hypothesis regarding the persistence of hepatocyte proliferation leading to liver cancer.

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