Transglutaminase Type II Plays a Protective Role in Hepatic Injury

Roberta Nardacci,* Oreste Lo Iacono,*[‡]
Fabiola Ciccocanti,* Laura Falasca,*
Maria Addesso,[§] Alessandra Amendola,*
Giorgio Antonucci,* Antonio Craxi,[‡]
Gian Maria Fimia,* Valentina Iadevaia,*
Gerry Melino,[¶] Luigi Ruco,[§] Guido Tocci,*
Giuseppe Ippolito,* and Mauro Piacentini*[†]

From the Istituto Nazionale per le Malattie Infettive,* IRCCS
"L. Spallanzani", Rome; Dipartimento di Biologia,[‡] Università di
Roma "Tor Vergata", Rome; Università degli Studi di Palermo,[‡]
Palermo; Dipartimento di Medicina Sperimentale e Patologia,[§]
Università di Roma, "La Sapienza", Rome Biochemistry
Laboratory IDI-IRCCS,[¶] c/o Dipartimento di Medicina
Sperimentale e Biochimica, Università di Roma, "Tor Vergata",
Rome, Italy; and the Medical Research Council,^{||} Toxicology Unit,
Leicester University, Leicester, UK

The up-regulation of "tissue" transglutaminase (TG2) gene has been shown to occur in various pathologies and can lead to severe liver injury; however, its role in the onset of liver damage has not yet been clarified. To address this issue, we have used two experimental settings: carbon tetrachloride (CCl₄)-induced liver injury in wild-type and TG2 knockout mice; and liver biopsies obtained from a large cohort of hepatitis C virus (HCV)-infected patients. Mice lacking TG2 failed to clear the hepatic necrotic tissue formed in response to prolonged CCl₄ exposure (5 weeks) and 60% of them died before the end of the treatment. By contrast, wild-type mice were able to recover after the toxic insult. CCl₄-treated TG2 null mice showed a derangement of the hepatic lobular architecture and a progressive accumulation of extracellular matrix (ECM) components and inflammatory cells which were not observed in the liver of control animals. Consistent with this protective role, we observed that TG2 levels were much higher (up to 15-fold) during the initial stages of liver fibrosis in HCV-infected individuals (METAVIR = F2) compared with uninfected controls, in which the enzyme protein localized in the hepatocytes facing the periportal infiltrate. By contrast, the enzyme levels decreased in the advanced stages (METAVIR = F3 and F4) and their localization was limited to the ECM. Our data demonstrate that TG2 plays a protective role in the liver injury by favoring tissue stability and repair. (*Am J Pathol* 2003, 162:1293–1303)

Transglutaminases are a family of intracellular and extracellular enzymes that catalyze Ca²⁺-dependent post-translational modification of proteins by establishing ε(γ-glutamyl)lysine crosslinkings and/or covalent incorporation of di- and polyamines. The establishment of these covalent cross-links leads to the oligomerization of substrate protein(s) which acquire peculiar features of resistance to breakage and chemical attack. Endoproteases capable of hydrolyzing these cross-links have yet to be identified in mammals.^{1–3}

The transglutaminase type II (TG2; E.C. 2.3.2.13) gene encodes a protein, which has a molecular weight of about 80 kd and is constitutively expressed in endothelial, mesangial, and smooth muscle cells.⁴ TG2 is a multi-functional enzyme that has been suggested to be involved in different biological processes including cell death, extracellular matrix stabilization, and signaling.^{5–8} In various mammalian cell lines TG2 overexpression leads to a higher incidence of spontaneous apoptosis and the clones resistant to TG2 overexpression show a marked priming for induction of cell death.^{9–12}

A complex role has been proposed for TG2 in degenerative diseases, which lead to severe tissue damage characterized by cell death and accumulation of insoluble protein aggregates both at intracellular and extracellular level.^{8,13,14} These diseases include liver damage occurring in various forms of hepatitis.^{15–17} Accordingly, we demonstrated that, in acute hepatic failure, such as in Budd-Chiari syndrome, the high incidence of cell death is associated with TG2 overexpression in the hepatocytes and with an abnormal release of the enzyme into the extracellular matrix (ECM).¹⁸

TG2 has also been suggested to play a role in the ECM organization in both normal and pathological conditions. A variety of ECM components, such as collagens, fibronectin, fibrinogen, laminin, nidogen, and transforming growth factor-α1 (TGF-α1) act as TG2 substrates.^{16,17,19–22} On the other hand, several inflammatory mediators, including TNF-α, TGF-β, interleukin (IL)-1β, and IL-6, involved in the fibrotic process and in apoptosis induction, are known to regulate TG2 expression in various biological settings.⁸

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Address reprint requests to Prof. Mauro Piacentini, Istituto Nazionale per le Malattie Infettive, IRCCS "L. Spallanzani", Via Portuense 292, Rome, Italy. E-mail: mauro.piacentini@uniroma2.it.

Evidence for TG2 having a role in hepatic fibrogenesis has been obtained by studying an animal model in which induction of fibrosis was achieved by carbon tetrachloride (CCl₄) administration,¹⁷ as well as by studying human patients with acute liver diseases.²³

Clinical relevance of chronic HCV infection is mostly related to its potential to induce liver damage either through a necro-inflammatory process or by direct induction.^{24–26} The prognosis of chronic hepatitis C, as indicated by the results of a large prospective cohort study, and the pathogenetic effect of alcohol, sex, and age is well established.^{25,27} The actual turning point in the course of disease is the development of cirrhosis and also the hepatocellular carcinoma, that not occurs in all patients. In fact, in a significant number of cases, fibrogenesis is absent even after many decades of infection.^{25,28}

In this work we have investigated the role of TG2 in the course of liver injury. To this aim we used two different approaches: 1) we have experimentally induced liver injury by intraperitoneal CCl₄ treatment of wild-type (WT) and TG2 knockout (KO) mice; 2) we have examined the expression of TG2 in liver biopsies obtained from 60 HCV-infected patients covering the various stages of fibrosis.

Materials and Methods

Animals

In the fibrosis model, studies were performed on C57BL/6 WT and C57BL/6 TG2 null mice.²⁹ TG2 genotype was confirmed for each mouse by genomic DNA isolation and polymerase chain reaction. Equal number (10 animals) of 10 week-old male WT and TG2KO mice received intraperitoneal injections of CCl₄ (0.5 µl/g/body weight) diluted 1:20 with mineral oil, twice a week. An identical group of both WT and TG2KO mice were injected with mineral oil in similar fashion and served as control. Three and five weeks after the injections the mice were weighed, anesthetized with Forane (Abbott Laboratories, Abbott Park, IL), and blood and liver samples were collected. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals.

Patients

We studied 60 patients, aged between 18 and 60 years, affected with chronic hepatitis C (CHC). All patients had a liver biopsy on enrollment in the study. All of the patients had abnormal ALT, were anti-HCV (EIA3), and HCV-RNA (Amplicor; Roche Diagnostic System, Basel, Switzerland) positive. All were negative for hepatitis B antigens (HBsAg), anti-human immunodeficiency virus (HIV), and non-organ-specific autoantibodies. Clinical and virological features are shown in Table 1. None had systemic disease which could increase connective tissue turnover. None had received antivirals or immunosup-

Table 1. Clinical and Virological Features of Enrolled Patients

Sex (m/f)	42/18
Age (years)	44.0 ± 12.6 (18–60)
Months since first ALT increase	54.3 ± 59.3 (6–240)
AST (IU)	63.9 ± 45.9 (21–238)
ALT (IU)	100.7 ± 73.2 (20–324)
GGT (IU)	43.9 ± 37.6 (12–164)
PLT (mmc × 1000)	194 ± 80 (63–424)
METAVIR	
A1, A2, A3	53%, 33%, 14%
F0, F1, F2, F3, F4	21%, 25%, 33%, 17%, 4%
HCV Viremia*	970 ± 773 (73–3870)
HCV Genotypes†	
1b	53%
Non 1b	47%

*Copies of genome equivalents/ml × 10³ by HCV-Monitor (Roche)

†By LiPA (Innogenetics).

pressants in the previous two years before evaluation. Patients who had either advanced cirrhosis (Child B or C), current active alcohol intake (>80 g/day), or intravenous drug abuse were excluded. Paraffin-embedded biopsies performed at least 2 years before inclusion in the study (mean 40.25 ± 8.01 months, range 26 to 49) were available for 8 of the 60 patients (3 with stable, 3 with improved, and 2 with progressive histological liver damage). The study protocol was approved by the local Ethical Committee and all patients provided written informed consent.

Serum Biochemical Parameters

Aspartate aminotransferase (AST), albumin (ALB), and cholinesterase (ChE) levels were determined using routine laboratory methods.

Virological Assessment

Qualitative and quantitative viremia were tested by HCV-Amplicor and HCV-Monitor (Roche Diagnostic System) with a sensitivity threshold of 102 (Amplicor) and 103 (Monitor) genome equivalents per ml. HCV typing was performed by a reverse-hybridization line probe assay (INNO-LiPA HCV; Innogenetics, Zwijndrecht, Belgium), based on variations of the 5' noncoding region.

Liver Histology

Animals

After sacrifice, the liver was excised and subdivided into fragments. One fragment was frozen for Western blot analysis and one fixed in buffered formalin and embedded in paraffin for histological examination and immunohistochemistry. Thin sections of paraffin-embedded liver were stained by hematoxylin and eosin and by Masson's trichrome.

Patients

Percutaneous liver biopsy was performed in all patients, using a disposable Menghini-type needle (Hepafix 1.6 mm; Braun Melsungen AG, Melsungen, Germany), as a part of the diagnostic routine for HCV infection. Histologically normal liver tissue obtained at laparoscopic surgery from 5 patients with uncomplicated colelithiasis served as control.

Liver biopsy specimens were divided into two parts. One fragment from each biopsy was fixed in buffered formalin and embedded in paraffin for histological examination and immunohistochemistry. The other part was processed for electron microscopy and Western blot analysis. Thin sections of liver biopsies were stained by hematoxylin and eosin, by Masson's trichrome and by Gomori. All specimens were examined for diagnostic purposes by one pathologist. At the end of the study the same pathologist blindly re-evaluated all biopsies and graded them according to METAVIR scores.²⁷ The second histological reading and score were taken as final diagnosis.

Immunohistochemical Analysis

For immunohistochemistry formalin-fixed, paraffin-embedded thin sections were used. Endogenous peroxidase activity was blocked by 3% H₂O₂. Monoclonal anti-TG2 antibody (NeoMarkers, Fremont, CA) diluted 1:25 or monoclonal anti-smooth muscle actin (SMA) (Dako A/S, Glostrup, Denmark) diluted 1:100 was applied to the sections followed by a biotinylated goat anti-mouse IgG. The immunoreaction product was revealed using aminoethylcarbazole (AEC) (Biogenex, San Ramon, CA) as chromogen substrates and 0.01% H₂O₂. Negative control staining was performed by omitting the primary antibody. Sections were counterstained in Mayer's acid hemalum.

Electron Microscopy Analysis

For standard electron microscopic examination small fragments of liver biopsies were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO₄, dehydrated in ethanol, infiltrated in propylene oxide, and embedded in Epon. Ultrathin sections were stained with 2% uranyl acetate and observed under a Zeiss EM 900 transmission electron microscope. For immunolabeling, samples of liver biopsies were fixed in 4% paraformaldehyde, dehydrated in ethanol, and embedded in LR-White resin. Ultrathin sections were incubated with the anti-TG2 (NeoMarkers) 1:25 for 1 hour at room temperature. After washing in PBS, samples were incubated for 45 minutes with goat anti-mouse IgG conjugated to 15 nm gold particles (BioCell, Cardiff, UK). Sections were then washed in PBS and stained with 5% uranyl acetate.

Western Blot Analysis

Western blot analyses of TG2 protein were performed in livers of WT and TG2 null mice, and in liver biopsies from

HCV-positive patients and healthy donors. The frozen tissues from hepatic biopsies were homogenized with lysis buffer (50 mmol/L Tris-HCl (pH 8) containing 120 mmol/L NaCl, 0.2 mmol/L EDTA, 1% NONIDET/IGEPAL, and protease inhibitor cocktail). Aliquots of total protein extracts (40 μ g) were run on 7.5% SDS-PAGE, in 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS and electroblotted onto nitrocellulose membrane overnight at 4°C in 25 mmol/L Tris, 192 mmol/L glycine, and 20% (v/v) MetOH. TG2-positive bands were revealed with monoclonal antibody specific for TG2 (NeoMarkers) and the appropriate secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad, Hercules, CA). Detection was achieved using a preformed streptavidin-horseradish peroxidase complex (Amersham Biosciences, Inc., Little Chalfont, Buckinghamshire, UK) and the signal was developed using ECL chemiluminescence detection system (Amersham Biosciences, Inc.).

Statistical Analysis

The liver sections of WT and KO treated mice stained by hematoxylin and eosin and by Masson's trichrome were semiquantitatively evaluated under code by three independent observers using a light microscope. To describe the inflammatory cells present and the fibrosis amount, an arbitrary value between 0 and 3 was assigned for each liver section, differentiating among the stages of liver pathology; the median values configuring a numerical score were used for statistical analysis. The liver sections of the 8 patients that underwent 2 biopsies were semiquantitatively evaluated under code by three independent observers using a light microscope without knowledge of either the clinical or histological diagnosis. To describe the degree of the enzyme expression on each specimen, an arbitrary value between 0 and 3 was assigned for each immunolabeled area as follows: 0, negative staining; 1, weak positive staining; 2, positive staining; 3, strong positive staining. Finally, data were averaged to median values configuring a numerical score for each liver biopsy specimen and used for statistical analysis. Continuous variables were expressed as mean $\pm$ SD ($\pm$ SD) or as median (range), as appropriate. The Kruskal-Wallis and the Mann-Whitney tests for non parametric data were used to compare medians, and the Pearson's correlation coefficient was used. Statistical significance was set at $P < 0.05$. The statistical analysis was performed by SPSS 10.0 for Windows (SPSS Science, Chicago, IL).

Results

The involvement of TG2 in liver diseases characterized by severe tissue alterations has been hypothesized by several authors^{8,15-19}; however, no direct demonstration of its role has been so far reported. In fact, previous studies have not directly addressed whether the accumulation of TG2 observed during hepatic injury was indeed playing a protective *versus* a pathogenetic role in the liver damage. In this study we used for the first time a

mice model in which the enzyme protein was ablated by homologous recombination, thus allowing the functional dissection of the TG2 in liver pathology.²⁹ Furthermore, we have analyzed TG2 expression in a large cohort of HCV-infected individuals representing all of the stages of the liver injury, including cirrhosis.

Effect of CCl₄ Treatment of TG2KO and Wild-Type Mice

TG2KO mice do not present an obvious embryonal phenotype, but they are very sensitive to stress-induced tissue damage.^{29–31} These findings prompted us to study the effect of TG2 ablation on liver injury. To this aim we injected for 5 weeks the hepatotoxin CCl₄ in two groups (10 animals) of WT and TG2KO mice. CCl₄ is a well-known hepatotoxin that induces a reversible acute centrilobular liver necrosis followed by a phase of tissue repair and fibrogenesis. The CCl₄ hepatotoxicity is dependent on the cytochrome P450 2E1 which generates the trichloromethyl radical, leading to lipid peroxidation and membrane damage.^{32,33} Subsequently, the Kupffer cells produce toxic mediators, inflammatory cytokines, and reactive oxygen intermediates, resulting in the injury of parenchymal cells.³⁴ Previous studies have suggested the involvement of TG2 in CCl₄-dependent liver injury.¹⁷ In agreement with these findings, we confirmed that WT mice accumulate TG2 protein as a consequence of prolonged CCl₄ treatment (Figure 1A). Although analysis of the serological markers of liver damage during the treatment with CCl₄, showed no statistically significant changes in TG2KO *versus* WT mice (Figure 1B), the TG2KO mice were more sensitive to the CCl₄ treatment compared to WT mice; in fact, 60% of the treated animals died before the end of the 5-week treatment (Figure 1C). To detect whether the differential response to CCl₄ was accompanied by specific alteration of the hepatic tissue, thin sections of liver fragments were stained with hematoxylin and eosin and with Masson's trichrome to evaluate the histopathological changes after CCl₄ treatment (Figure 2). KO mice show a more evident inflammatory response at 3 and 5 weeks, which is paralleled by an increased fibrosis (Figure 1D). The considerable accumulation of ECM observed in the liver in KO mice is statistically significant ($P = 0.007$) when compared with control animals (Figure 1D).

The morphological analysis shows that, at the end of 5-week treatment, the liver of WT mice displayed a low number of infiltrating cells visible in intralobular areas (Figure 2A) accompanied by a limited ECM deposition in the periportal areas (Figure 2C). By contrast, the TG2KO mice survived the treatment, showed a drastic alteration of the liver architecture due to a widening of the interlobular space in all lobular areas. In addition, the presence of a larger number of inflammatory cells in the hepatic parenchyma was observed (see insert in Figure 2B) and the portal tract showed massive enlargements surrounded by concentric accumulation of ECM (Figure 2D). These data demonstrate that while WT mice are able to rescue the liver architecture after 5 weeks of CCl₄ injection,

the surviving TG2 null mice show evident liver injury characterized by the presence of necrotic tissue and abnormal ECM accumulation. In a subsequent set of experiments, we determine whether activation of hepatic stellate cells (HSCs) contributes to the ECM accumulation observed in TG2 null mice, by using anti-smooth muscle actin (SMA) antibodies.³⁴ A significantly increased number of activated HSCs in both periportal and pericentral liver regions of TG2KO mice were detected in comparison with WT mice (Figure 2, E and F). Considering that TG2 absence renders the hepatic tissue more sensitive to toxic insults, the enzyme induction observed in the WT mice in the early phase of the treatment might be envisaged as an important element of liver stress response program.

Analysis of TG2 Expression in Liver Biopsies of HCV-Infected Individuals

To confirm and extend the above reported findings in human liver pathology characterized by severe tissue damage and scarring processes we investigated the expression of TG2 during HCV-induced liver hepatitis. In fact, an increasing cause of liver disease worldwide is HCV infection.²⁴ Thus, we studied the TG2 expression profile in a cohort of 60 HCV-infected individuals encompassing the different grading and staging of liver damage according to the METAVIR score.²⁷ Notably, we found a marked increase of TG2 protein levels in the livers of HCV-infected patients when compared with uninfected controls (Figures 3 and 4). To verify whether there is a direct correlation between the progression of the disease and the accumulation of TG2 protein in the liver, we quantified TG2 protein levels in uninfected liver homogenates compared with those obtained from HCV-infected biopsies according to the METAVIR fibrosis score. An average increase of 3- to 4-fold in TG2 levels was detected in liver homogenates of non-fibrotic (METAVIR = F0,F1) HCV-infected patients when compared with controls (Figure 3A). The accumulation of TG2 in the liver tissue reached its maximum level at fibrotic stages without cirrhosis (15-fold increase *versus* uninfected controls; METAVIR = F2). This increase is statistically significant and correlates with the METAVIR fibrosis score ($P = 0.003$). By contrast, the more advanced stages of the disease were characterized by a marked decrease in TG2 levels when compared with the initial fibrotic stages (METAVIR = F3,F4). Analysis of 8 patients who underwent two serial biopsies confirmed the correlation existing between the HCV disease stage and TG2 expression ($P = 0.002$). In fact, progressive disease was associated with an increased TG2 expression, while a decrease in fibrosis METAVIR index in the same patient was paralleled by a concomitant reduction in TG2 expression (Figure 3B).

To get further insights into the role of TG2 during HCV-induced liver injury, a detailed immunolocalization analysis of TG2 was performed in liver sections from HCV-infected patients (Figure 4). As far as the necro-inflammatory activity (periportal and intralobular) is con-

Figure 1. Effect of CCl₄ treatment and serum marker in WT and TG2 null mice. **A: Upper panel,** Western blot analysis of TG2 protein expression; **lower panel,** densitometric analysis of western blot. A representative experiment out of three is shown. The enzyme, as expected, is not present on livers of TG2KO mice treated with CCl₄, treated only with mineral oil (Oil) and untreated (Ctr). **B:** Serum markers of untreated (Time 0) and CCl₄-treated mice for 3 weeks and for 5 weeks expressed as percentage with the respect to the controls. Serum alanine transferase (ALT), albumin (ALB), and cholinesterase (ChE). **C:** Survival curve of CCl₄ treated animals. While all WT mice survived for the 5 weeks of treatment; only 40% of the TG2 null treated for 5 weeks survived till the end of experiment. **D:** Liver histopathological analysis of WT and KO treated mice. TG2KO mice show higher number of inflammatory cells at 3 weeks and 5 weeks of treatment. This phenomenon is also accompanied by a considerable accumulation of ECM material; the fibrosis increase is statistically significant ($P = 0.007$) if compared to WT treated mice.

cerned, we detected TG2 immunostaining preferentially localized in the altered hepatic parenchyma bordering the portal tract (Figure 4, A and B). Interestingly, the enzyme accumulated in hepatocytes in contact with the periportal infiltrate (Figure 4D) and in areas where lymphocytes (or inflammatory cells) infiltrated the hepatic parenchyma (Figure 4C). In advanced liver disease showing more prominent fibrosis, TG2 was detected in the amorphous cirrhotic tissue (Figure 4, E and F). Immunostaining performed on HCV-negative liver biopsies

showed that TG2 was localized only in endothelial cells where the enzyme is constitutively expressed (not shown).

Finally, we performed a detailed analysis of TG2 hepatic localization during fibrotic tissue formation by immunogold technique at the transmission electron microscope. At earlier stages of hepatic injury, TG2 is localized in the enlarged space between neighboring cells (Figure 5, A and B) where fibrillar material deposition is also visible (Figure 5B). Enhanced levels of TG2 were de-

Figure 2. Liver histology and immunohistochemistry of WT and TG2KO mice after 5 weeks of CCl_4 treatment. **A** and **B:** Hematoxylin and eosin stain. **A:** WT mice show a mildly altered liver histology, and some inflammatory cells are visible in intralobular areas and in sinusoidal spaces. **B:** TG2KO mice show a lower hepatocyte adhesion and a massive presence of inflammatory cells in the dilated spaces in each region of the hepatic parenchyma (arrows). The insert shows an higher magnification of the same section pointing out inflammatory cells (arrow) and red blood cells (*). **C** and **D:** Masson's trichrome stain. **C:** WT mouse shows evident fibrotic tissue accumulation in the liver periportal areas (arrows). **D:** TG2KO mice show a prominent accumulation of eosinophilic material in some areas of hepatic parenchyma (double arrowheads), the portal tract displayed an important enlargement that is surrounded by fibrotic tissue (arrow). **E** and **F:** Immunohistochemical localization of smooth muscle actin (SMA) indicating the activated hepatic stellate cells (HSCs). **E:** WT mice show the presence of some activated HSCs near the sinusoidal spaces and particularly close to periportal regions (arrows). **F:** TG2 null mice displayed a much higher number of activated HSCs and particularly in periportal areas where also some hepatocytes are labeled (arrows). Scale bars, 100 μm .

tected both in the hepatocytes and ECM in patients with inflammatory infiltrate penetrating liver parenchyma (Figure 5, C and D), confirming the light microscopy observations. Gold particles were localized along the plasma membrane of hepatocytes facing the infiltrating cells and

in correspondence with fibril bundles released by hepatocytes in the same area (Figure 5D). With fibrosis progression and formation of larger ECM deposits, TG2 was exclusively found in the amorphous cirrhotic regions associated with fibril bundles (Figure 5, E and F).

Figure 3. Western blot analysis of TG2 protein expression in the liver of HCV-infected patients and TG2 expression in repeated liver biopsies. **A:** TG2 expression in liver biopsies from patients with different grading of damage, according to the METAVIR fibrosis score, was compared to homogenate obtained from control uninfected liver. Densitometric analysis, by NIH Image, revealed an increase of TG2 protein levels in liver homogenates of HCV-infected patients at early fibrosis stages (METAVIR F0–F2) that is statistically significant ($P = 0.003$), and a decrease at advanced stages (METAVIR F3, F4). Aliquots of 40 μ g were analyzed in each lane and the amount of protein was confirmed by re-staining the same filters with anti-actin IgGs (not shown). **B:** Time course analysis of TG2 expression in repeated liver biopsies in the same patients. **Upper panel** shows the METAVIR fibrosis score at first and second biopsy; **lower panel** shows the TG2 immunoreaction product on each biopsy. Patients 1, 2, and 3 do not show any variation in liver fibrosis between the first and second biopsy and also TG2 expression remained unchanged. Patients 4 and 5 show an higher stage of fibrosis at second biopsy which correlates with an increased TG2 expression. Patients 6, 7, and 8 clearly demonstrate a reduction of fibrosis paralleled by a lower expression of TG2. A statistically significant correlation has been found between the two parameters ($P = 0.002$).

Discussion

Our studies demonstrate that TG2 plays a critical role in the course of hepatic repair following a prolonged toxic injury. Analysis of TG2KO mice indicated that the primary

consequence of lack of TG2 function(s) is the defective clearance of necrotic cells associated with production of ECM; these pathological events drastically compromise the capacity of TG2 null mice to survive a toxic injury. In keeping with these findings is the TG2 expression profile detected in HCV-dependent hepatitis. In fact, we found that the increased TG2 levels as well as its distribution are strictly related to the different stages of liver damage. TG2 increases early during liver injury and its intracellular/extracellular localization varies according to the stage of the disease. In HCV-infected patients, the enzyme levels are particularly high (15-fold increase) during the stage of more active liver fibrogenesis and inflammatory process. While in stages characterized by prominent ECM deposition, TG2 expression decreased and was confined to the ECM. It is interesting to note that in the absence of TG2, mice develop an increased inflammatory response associated with an altered liver architecture, thus suggesting a role for the TG2 protein as a general protective element in hepatic homeostasis. It is interesting to note that both CCl₄-treated mice and HCV-infected patients share the maximum increase in TG2 levels during the early stages of the liver injury (Figures 1A and 3A).

Although the progression of hepatitis C is widely described, the cellular mechanisms underlying liver injury are not fully understood. The presence of inflammatory cells in the hepatic parenchyma has been interpreted as evidence for immune-mediated damage.²⁶ Moreover, a study performed on HCV-positive patients showed that the rate of progression to cirrhosis was accelerated in patients whose initial biopsies showed a high grade of necro-inflammation and septal fibrosis, thus confirming the pivotal role of lymphocyte derived cytokines and chemokines in inducing ECM formation.³⁵ In keeping with this hypothesis, at non-fibrotic stages, TG2 protein is almost exclusively localized in the hepatocytes bordering the periportal infiltrate, thus suggesting that its expression is induced by soluble factors released by the infiltrating cells. Several cytokines, such as TNF- α , IL-1 β , TGF- α , and IL-6 are secreted in response to liver injury.^{8,17} These molecules have been shown to be responsible for TG2 induction in various cells under different biological conditions.^{8–36} The pro-inflammatory mediator TNF- α , for example, is known to be expressed during hepatic injury, regeneration, and fibrosis, as well as to modulate TG2 activity in cultured liver cells.³⁶ It is tempting to hypothesize that by crosslinking extracellular proteins^{8,19} and/or by increasing cell-ECM adhesion^{22,37} TG2 might counteract the infiltration of inflammatory cells in the liver parenchyma during HCV pathogenesis in early stages of the disease.

The hypothesis that TG2 may play a protective role in the acute phases of liver injury consequent to HCV infection is also supported by the finding that the stages characterized by severe fibrosis show a marked decrease of TG2 levels (threefold, compare F2 with F3 and F4, in Figure 3A). These findings are supported by the fact that TG2KO mice display an altered response to liver injury characterized by loss of cell-to-cell adhesion associated with an enhanced inflammation and ECM deposi-

Figure 4. Immunohistochemical localization of TG2 in hepatitis C virus infected liver. Masson's trichrome stain of mild, non fibrotic hepatitis C virus infected liver shows hepatic parenchyma bordering a pathologically altered portal tract. Inflammatory cells are present and also fibrotic tissue accumulation (A). A consecutive section of the same specimen has been stained with anti-TG2 antibody; positivity is present on hepatocytes bordering the periportal tract (B) and is especially high on liver areas where piecemeal necrosis is present as is visible in a magnification of the same specimen (C, arrows). The hepatocytes bordering the periportal infiltrate, and in direct contact with lymphocytes, show clearly TG2 expression (D). Bile duct epithelial (arrowhead) and endothelial cells (double arrowhead) are both stained (C and D). Masson's trichrome infected liver with an advanced stage of fibrosis stain shows hepatic parenchyma quite normal and an area of fibrosis accumulation (E). A consecutive section stained with anti-TG2 antibody shows that positivity is limited to extracellular matrix accumulation while the hepatocytes are quite negative (F). Scale bars, 100 μ m (A, B, E, F); 50 μ m (C, D).

tion. According to this view, TG2 induction must be considered as part of stress-induced damage and an important member of the hepatic tissue response to the progression of liver pathogenesis.

The ultrastructural analysis of infected liver biopsies confirmed the association of TG2 protein with the plasma membrane of hepatocytes and fibrillar bundles localized in the enlarged spaces between neighboring cells sup-

Figure 5. TG2 immunogold localization in HCV infected livers at different stages of hepatic injury. Parts **B**, **D**, and **F** are higher magnification of the squared areas respectively in pictures **A**, **C**, and **E**. **A** and **B**: In the early stage of hepatic injury, TG2 is present in the dilated spaces (*) between the lateral domain of neighboring hepatocytes (**H**), and, as shown at higher magnification (**B**) gold particles are associated with the presence of fibril bundles. **C** and **D**: In hepatic parenchyma facing periportal inflammatory areas and in direct contact with lymphocytes (**L**), TG2 was localized along the plasma membrane of hepatocyte (**H**) and, as shown in **D**, in association with initial deposits of fibrillar material (**arrowheads**). **E** and **F**: As the liver tissue (*) becomes fibrotic, gold granules appear almost exclusively localized in areas of ECM deposition, always in correspondence of fibril bundles. Original magnifications: **A**, **C**, and **E**, $\times 6000$; **B**, **D**, and **F**, $\times 40,000$.

porting its role in cell adhesion. By contrast, TG2 is almost exclusively localized in the ECM in advanced liver injury when the fibrosis is predominant.

Several cellular mechanisms are involved in TG2 externalization in the ECM^{8,18} and a novel function for cell

surface TG2 as an integrin-associated adhesion co-receptor for fibronectin has recently been proposed.^{22,37} This function is distinct from and independent of its crosslinking activity, but depends on its stable non-covalent association with integrins. The interaction of TG2-

integrins promotes cell adhesion by creating an additional binding site within fibronectin for its interaction with integrins.^{22,37} In support of this extracellular function, we demonstrated that TG2 null mice treated with CCl₄ showed a marked reduction of hepatocytes adhesion associated with a prominent presence of inflammatory cells penetrating hepatic parenchyma. In keeping with this assumption, it is worth mentioning that IL-10 null mice develop a TG2 null-like phenotype when treated with CCl₄ (ie, accelerated fibrosis and proinflammatory status).³⁸ IL-10 is a well known anti-inflammatory cytokine synthesized during liver inflammation and possesses monocyte/macrophage de-activating properties. It is interesting to note that the hepatic macrophages secrete a range of pro-inflammatory cytokines including IL-6 that is known to regulate TG2 expression.^{8,38}

Another pathway by which TG2-catalyzed protein crosslinking might play a regulatory role in liver repair/injury is through the activation of latent TGF- β 1,³⁹ which is known to be involved in various physiological and pathological processes such as inflammation, tissue repair, autoimmune disease, and hepatic fibrogenesis.^{40,41}

In conclusion our data suggest a complex role for TG2 in liver damage. Its ablation in a mouse model leads to an impaired liver regeneration after injury associated with an increased inflammatory response, and to an abnormal tissue architecture associated with a reduced survival. This evidence support the hypothesis that the increased TG2 levels, detected at early stages of HCV-induced liver damage, plays a protective role in maintaining liver architecture by counteracting the consequence of inflammatory response. Taken together these findings for the first time demonstrate that TG2 expression is an essential element of stress-induced hepatic program conferring resistance to damage.

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