Improvement in Dissolution of Liver Fibrosis in an Animal Model by Tetrathiomolybdate

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The background for this study is that we have observed some improvement in cirrhosis in Wilson's disease patients treated with the anticopper medicine, zinc, and another anticopper drug, tetrathiomolybdate, has completely prevented hepatic fibrosis in the carbon tetrachloride mouse model. We hypothesize that in existing cirrhosis, there may be a fine balance between fibrosis formation and fibrosis dissolution, which may be pushed in the direction of dissolution by anticopper drugs. Thus, in this study, we produced hepatic fibrosis in mice by treatment with carbon tetrachloride, then gave half the fibrotic mice tetrathiomolybdate for 3 months, while the other half of the fibrotic mice received nothing for 3 months and served as controls. Tetrathiomolybdate caused a dramatic and significant reduction in fibrosis as measured by hydroxyproline (the major amino acid constituent of collagen) levels, almost back to baseline levels, compared to controls, who had only a slight and nonsignificant reduction. It is clear from this animal study that dissolution of preexisting fibrosis can be strongly catalyzed by lowering copper levels with tetrathiomolybdate. It now becomes important to evaluate whether this approach will work in the human epidemic of cirrhotic disease resulting from diseases such as alcoholism, nonalcoholic steatohepatitis, and hepatitis C. Exp Biol Med 234:662–665, 2009

Key words: anticopper therapy; therapy of liver fibrosis; carbon tetrachloride liver injury; hydroxyproline; ceruloplasmin

Introduction

The concept is prevalent that cirrhosis is not generally reversible, that once cirrhotic changes occur in the liver they are permanent. However, we have been impressed by our own personal observations, plus a publication by Marcellini *et al.* (1), that over years of zinc therapy, cirrhotic livers in Wilson's disease do gradually improve. In this case since the main effect of zinc is probably to simply prevent the reoccurrence of toxic copper, the liver is presumably causing its own improvement, probably by a slow process of remodeling the fibrotic tissue in favor of more normal liver tissue.

We presume this remodeling process favors, at least slightly, a fibrosis dissolution process versus a fibrosis enhancing process, the latter of which is no doubt dependent on the transforming growth factor beta (TGF_β) pathway of fibrosis production (2). In the bleomycin model of pulmonary fibrosis (3, 4), in the carbon tetrachloride model of hepatic fibrosis (5), and in the bile duct ligation model of hepatic fibrosis (6), we have repeatedly shown inhibition of TGF_β while largely preventing fibrosis, by lowering copper availability with tetrathiomolybdate (TM). Given these results, it seems possible TM therapy would tip the balance further in terms of enhancing fibrosis dissolution in preexisting hepatic fibrosis.

In this study, as in previous studies of TM therapy, we use serum ceruloplasmin (Cp) as a surrogate marker of copper availability. Serum copper can't be used for this purpose because it builds up in the blood during TM therapy as a complex with TM and albumin (7). Ceruloplasmin, a molecule rich in copper, is secreted into the blood by the liver dependent upon the availability of copper (8). In animal model studies we have found that reducing Cp to 30–50% of baseline reduces copper availability enough to inhibit the synthesis or activity of many cytokines, including TGF_β, while not producing clinical copper deficiency, the first indication of which is bone marrow depression (3–6).

In this study, we have carried out experiments to determine whether TM therapy would improve the dis-

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[2] Conflict of interest: The University of Michigan has licensed the antifibrotic and anti-inflammatory uses of TM to Pipex Therapeutics, Inc., Ann Arbor, MI. Dr. Brewer has equity in and is a paid consultant to Pipex Therapeutics, Inc.

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solution of hepatic fibrosis produced by prior treatment of mice for 12 weeks with carbon tetrachloride (CT).

Materials and Methods

Mice. The mice were Balb/c 20 gram females purchased from the Jackson Laboratory, Bar Harbor, ME. The mice were housed in the University of Michigan Unit for Laboratory and Animal Medicine facility and treated in accordance with a protocol approved by the University of Michigan Institutional Animal Care and Use Committee. The protocol is in compliance with criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Mice were assigned to groups randomly, were provided Purina mouse chow (containing more than adequate copper) and water *ad libitum*, and kept on a 12:12-hour light:dark cycle.

Carbon Tetrachloride (CT) Treatment. The CT was purchased from Sigma Chemical Co., St. Louis, MO. Mice in the CT treatment groups received intraperitoneal injections of 0.05 ml/Kg of body weight in 200 μl of olive oil twice per week for 12 weeks. Control animals received 200 μl of olive oil alone by intraperitoneal injection twice per week for 12 weeks.

Tetrathiomolybdate (TM) Treatment. The TM was synthesized by Dr. Dimitri Coucouvanis of the University of Michigan Chemistry Department, whom we thank. The TM was given in drinking water at concentrations of 0.02 mg/ml to 0.03 mg/ml, adjusted to keep the animals' ceruloplasmin between 30% and 50% of their baseline.

Experiments. In a preliminary experiment, 15 mice were given CT for 12 weeks, and 8 served as controls. Then 8 of the CT treated mice received TM for 12 weeks and the other 7 CT treated mice received nothing and served as CT treated controls. At this second 12 week point all 23 mice were sacrificed and liver hydroxyproline levels measured. (Hydroxyproline, the major amino acid constituent of collagen, is a marker of the severity of fibrosis.)

In a second, definitive experiment, 25 mice were treated with CT, while 12 mice served as controls. At 12 weeks of treatment, 5 CT treated and 5 control mice were sacrificed, and liver hydroxyproline measured to determine the starting point level of fibrosis before TM therapy. Then 10 CT treated mice began treatment with TM, while 10 CT treated mice received no further treatment, and served as CT treated controls. Seven completely untreated mice served as controls. After 3 months of TM therapy, all mice were sacrificed, and liver hydroxyproline levels determined.

Assays. We use serum ceruloplasmin (Cp) as a surrogate marker of copper status. The Cp level was assayed by its oxidase activity (9). The assay of hydroxyproline content of the liver was performed as previously described (3) for the second study and the data reported as hydroxyproline per g of wet weight of liver. For the first, preliminary study, the tissue was dried first in a vacuum oven at 60°C, and then the assay carried out as in reference 3. These preliminary results were expressed per g of dry weight of liver. Mouse serum interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα) were assayed using Mouse Th1/Th2 Cytokine Kit by BD cytometric bead array (BD Biosciences, San Jose, CA) on the plasma of mice at the time of sacrifice. Serum alanine amino transferase (ALT) assay was performed using a quantitative colorimetric method commercially available from Sigma Diagnostics (Procedure 505), St. Louis, MO.

Statistical Analysis. For comparison of means, analysis of variance was used.

Results

In the preliminary experiment, the mean hydroxyproline levels in the seven CT treated controls and the eight CT/TM treated animals were significantly higher than the mean of the eight untreated controls, showing that the CT treatment elevated hydroxyproline levels (data not shown). The mean of the CT/TM treated animals is lower than the CT controls, who received nothing during the 12 weeks of TM treatment in the CT/TM animals, but barely escapes statistical significance ($P = 0.058$). (Data not shown.) This preliminary study was done to show proof of concept.

In the second, definitive experiment, the number of animals was increased to help show statistical significance. After CT treatment for 12 weeks, five CT treated animals were sacrificed and the liver hydroxyproline levels compared to five untreated controls. The results are shown in Figure 1. The CT treated animals had almost six times as much hydroxyproline as non-treated controls, a result that is significant at $P < 0.0001$.

In this experiment, the 20 remaining CT treated animals were divided into two groups of 10 each, and one group of 10 was treated with TM for three months (CT/TM treated), while the other 10 CT treated animals received nothing and served as CT treated controls. Seven animals, completely untreated, served as untreated controls. After 3 months, all the animals were sacrificed and the hydroxyproline levels assayed. The results are shown in Figure 2. The CT treated animals that were untreated for the three months showed a mean hydroxyproline value of 0.225 mg/g wet weight (the leftmost column of Fig. 2), slightly down from the mean value three months earlier which was 0.292 (left column of Fig. 1). However, the CT/TM treated mice showed a marked and statistically significant reduction, compared to CT treated controls, to a mean value of 0.095 mg/g wet weight ($P = 0.009$) (comparison of leftmost and middle columns of Fig. 2). This mean value was not significantly different than the mean of completely untreated controls, which was 0.058 mg/g wet weight ($P = 0.48$) (comparison of middle and rightmost columns of Fig. 2).

The results of plasma cytokine assays at the time of

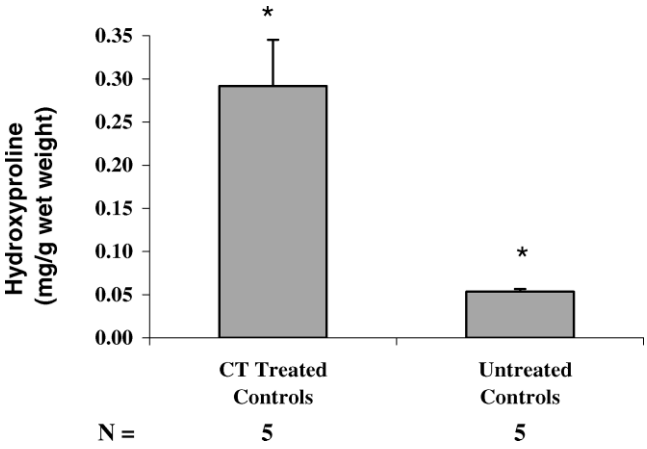

Figure 1. Mean and SE of hydroxyproline levels expressed as mg/g wet weight of liver in the livers of mice of the definitive experiment (Experiment 2) who received CT for 12 weeks (CT treated) or served as untreated controls. The mean level of hydroxyproline in CT treated animals was significantly elevated compared to the mean of untreated controls (* $P = 0.0001$).

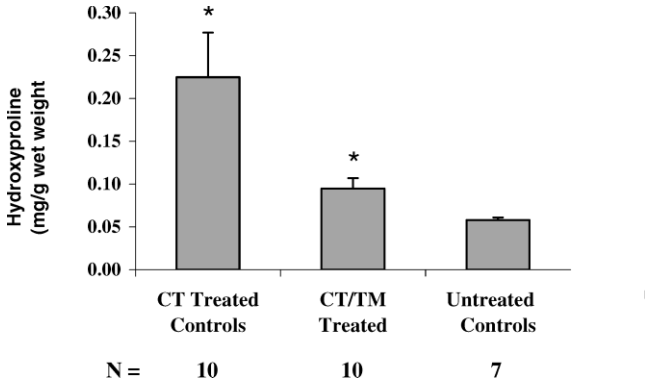

Figure 2. Mean and SE of hydroxyproline levels expressed as mg/g wet weight of liver tissue in the livers of mice of the definitive experiment (Experiment 2) who received CT for 12 weeks then nothing for 3 months (CT treated controls) or CT for 12 weeks then TM for 3 months (CT/TM treated) or were completely untreated (untreated controls). The mean of CT/TM treated animals was significantly lower than the mean of CT controls (* $P = 0.009$) but not significantly different than the mean of untreated controls ($P = 0.48$).

sacrifice of the mice after the completion of experiment 2, are shown in Figure 3. Th-1 cytokines TNFα and IFN-γ and IL-2, are not significantly different in the three groups. Interestingly, the Th-2 cytokines IL-4 and IL-5 seem to be significantly decreased from controls by prior CT treatment, but significantly restored toward the normal range by TM treatment (Fig. 3).

Discussion

Tetrathiomolybdate was originally developed for treating the neurologic presentation of Wilson's disease (7, 10–13). Its anticopper mechanism involves formation of a three-way complex with protein and copper which is very stable (14–18). Given with food, it combines with food protein and food copper, as well as copper in endogenous secretions, and prevents its absorption. Given away from food, it is well absorbed, and in the blood combines "free" copper with albumin. The copper in this complex is not available for cellular uptake. In this way the so-called "free" copper of the blood, which is defined as the non-ceruloplasmin and non-TM bound copper, is largely unavailable for cellular uptake and use. The copper in ceruloplasmin is covalently bound, is not available for easy exchange, and accounts for about 90% of blood copper in most mammals including the human and the mouse. The "free" copper is loosely bound to albumin and small molecules, and this pool is expanded in Wilson's disease and causes toxicity. In non-Wilson's people and in mice, the free copper is the copper available to activate cytokines involved in fibrosis, inflammation, and in autoimmune diseases. With TM treatment, the free copper levels can be drastically reduced.

In inflammatory disease mouse models, we have shown that TM prevents much of the liver damage from acetaminophen (19) and the heart damage from doxorubicin (20). In immune-modulated models, we have shown that TM prevents much of the hepatitis of concanavalin A treatment (5), the arthritis from bovine collagen II injection (21), spinal cord damage in a multiple sclerosis model (22), and prolongs the development of diabetes in the non-obese diabetic mouse model, a model of type I diabetes in the human (23).

The cytokines shown in Figure 3 were measured because we expected the Th-1 inflammatory cytokines (IL-2, IFN-γ and TNFα) to be increased by CT treatment, and for TM to inhibit this increase. However, we saw no significant effect of either CT treatment or TM therapy on these cytokines (Fig. 3). Interestingly, CT treatment significantly inhibited the Th-2 cytokines IL-4 and IL-5, and TM had a significant effect in restoring them towards normal (Fig. 3). Th-2 cytokines may be secreted to help quell the inflammatory response, so perhaps CT inhibits the Th-2 response while TM helps normalize it.

Our animal model studies have also shown that TM treatment in mouse models of fibrosis, such as pulmonary fibrosis from bleomycin (3, 4), hepatic fibrosis from carbon

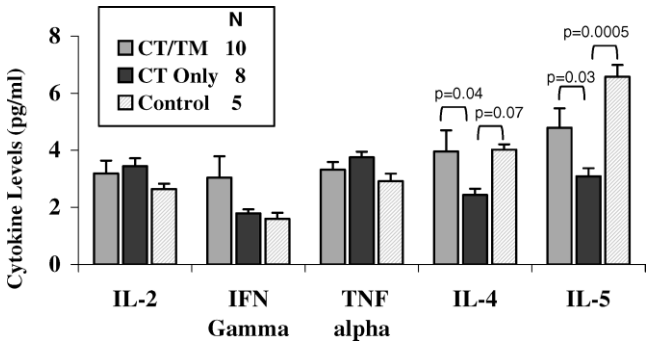

Figure 3. Plasma cytokine data at the time of sacrifice of some of the animals of Experiment 2. With both IL-4 and IL-5, prior CT treatment seems to decrease levels, and TM treatment restores levels to more nearly normal.

tetrachloride treatment (5), and hepatic fibrosis from bile duct ligation (6), prevents much of the fibrosis. But this study is different, in that it shows that TM treatment after establishment of hepatic fibrosis can greatly speed the resolution of the preexisting fibrosis (Fig. 2).

We should make it clear that enhanced dissolution of fibrosis by TM, as shown here by enhanced reduction of hydroxyproline levels, does not prove that TM will improve cirrhosis, which is a histologic diagnosis. However, excessive fibrosis is a hallmark of cirrhosis, so this study is encouraging that TM will benefit cirrhotic disease.

We believe these results are potentially very exciting. They suggest that the balance of fibrosis in the liver can be strongly tipped towards dissolution by lowering copper levels. This has been suggested to us by noting gradual histologic improvement in cirrhosis in Wilson's disease patients long treated with zinc (1), which of course would lower copper levels. If these observations are applicable to human cirrhotic diseases in general, it could open up treatment for a host of patients with well established hepatic fibrosis, such as patients with cirrhosis from alcoholism, hepatitis C, non-alcoholic steatohepatitis, and so on.

We currently have a small double blind clinical trial of TM proceeding in primary biliary cirrhosis. The extent of benefit in this autoimmune disease will probably depend primarily upon the inhibition of the autoimmune and inflammatory aspects of the disease, therapeutic properties which TM also has, as discussed above. But a better test of the more pure resolution of cirrhosis might come from diseases where the inciting process has stopped or abated, such as in alcoholics who are abstaining, or hepatitis C with little or no viral load. We would like to see such testing begin as soon as possible.

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