Commentary

A Murine Model of Alcoholic Cardiomyopathy

A Role for Zinc and Metallothionein in Fibrosis

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Chronic alcohol use in humans results in functional changes and pathology in multiple organ systems. Particularly affected are the liver, the heart, and the pancreas. Alcohol-induced disease of these organs often is a significant clinical problem, resulting in serious or fatal illness, particularly in cases of chronic alcohol abuse (defined as greater than 90 g of alcohol per day for 5 years or more). In humans, such exposure to alcohol typically results in a form of dilated cardiomyopathy that is characterized by reduced contractility, ventricular dilatation, cardiomyocyte apoptosis, and fibrosis, often progressing to heart failure.¹ Cardiac fibrosis involves fibroblast proliferation and transdifferentiation to myofibroblasts, extracellular matrix remodeling, and stiffening of the ventricular wall. Because fibrosis exacerbates the decline of ventricular function and contributes to dilatation and progression to failure, it represents an attractive therapeutic target.² To date, features of alcoholic cardiomyopathy (ACM), notably fibrosis, are not reproducibly recapitulated in animal models. In this issue of *The American Journal of Pathology*, Wang and colleagues³ detail a novel model of ACM and investigate the effects of metallothionein (MT) and zinc on ACM and myocardial fibrosis.

Alcohol affects the heart both directly and indirectly. Direct effects involve perturbation of the structure/function and/or metabolism of cardiac constituent cells. Indirect effects are mediated by other organs and by neurohormonal factors. Although the liver is the primary site of alcohol metabolism, alcohol also acts on the myocardium via direct effects on cellular metabolism. Myocardial cells metabolize ethanol via alcohol dehydrogenase to produce acetaldehyde, which enhances release of catecholamines and directly impairs cardiac function.⁴ Alcohol also interferes with excitation-contraction coupling by affecting the calcium transit.⁵ Ethanol can combine with fatty acids to produce fatty acid ethyl esters that damage mitochondria and membranes, and alcohol en-

hances lipid peroxidation, which damages cellular membranes. Cardiac muscle from alcohol-fed animals has a reduced capacity for oxidative metabolism and increased glycolysis, resulting in a more acidic intracellular environment. Alcohol is known to alter mitochondrial metabolism and to enhance production of oxygen-derived free radicals, including superoxide ($O_2^{\cdot-}$), and thus has direct effects on generation of reactive oxygen species.^{2,6} Acute alcohol exposure reduces the levels of the myofibrillar proteins and reversibly reduces cardiac contractile function while chronic exposure to alcohol leads to further irreversible reduction of myofibrillar protein.⁷ Chronic alcohol administration increases the probability of fibrillation⁵ and affects excretion and tissue levels of metal ions.⁸ Thus, alcohol exposure to the heart generally leads to decreased myocardial contractile function, cellular damage, and increased oxidative stress.

Effects of Alcohol on Minerals

In both the human liver and heart, alcohol use is associated with perturbation of metal ion homeostasis. It is not known whether this is a primary effect of alcohol or an indirect result of tissue damage, but serum zinc deficiency is common among patients with idiopathic cardiomyopathy.^{9,10} In both liver and heart, chronic alcohol use results in reduced levels of zinc.¹¹ In the liver, where this is better studied, zinc depletion occurs in association with high tissue levels of copper, iron, and manganese and increased fibrosis.⁸ Numerous studies have reported that low zinc levels associate with alcohol abuse and correlate with the severity of liver cirrhosis and mortality.^{8,12-14} Anttinen and colleagues¹⁵ found that zinc supplementation reduces collagen accumulation and fibrosis in a model of carbon tetrachloride-induced liver injury. This result was confirmed by subsequent investigations.^{16,17} Elsherif and colleagues¹⁸ showed that liver fibrosis resulting from carbon tetrachloride treatment for 4

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weeks was reversible by withdrawal of the carbon tetrachloride. However, damage resulting from an 8-week course of carbon tetrachloride treatment induced irreversible damage and fibrosis associated with reduced hepatic MT levels. These investigators went on to show that, in mice deficient for MT-I and -II (MT-KO), the 4-week treatment regimen induced irreversible liver damage and fibrosis. Importantly, adenoviral delivery of the human MT II gene to the liver reversed fibrosis and activated hepatocyte regeneration in both MT-knockout (KO) (4 weeks) and wild-type mice (8 weeks) with irreversible fibrosis. The increased MT activity was associated with increased collagenase activity, suggesting that the MT II gene therapy activated anti-fibrotic pathways in the damaged livers. This was the first study to implicate MT in the modulation of fibrosis, but whether this acts directly or via the reactive oxygen species scavenging activity of MT was not addressed. Until now, there has been no data bearing on a role for MT in cardiac fibrosis.

Zinc, copper, iron, and manganese play important roles as co-factors for several of the enzymes involved in collagen synthesis/degradation and extracellular matrix remodeling. Low zinc levels enhance prolylhydroxylase activity and inhibit collagenases, favoring collagen deposition.^{19,20} Also, low zinc levels impair superoxide dismutase activity²¹ that would normally counter increased generation of free radicals subsequent to alcohol exposure. Zinc and copper are required co-factors for the activity of matrix metalloproteinases (MMPs), which are critically involved in extracellular matrix remodeling. MMPs are grouped into four major categories; collagenases (eg, MMP1, MMP13), gelatinases (eg, MMP2, MMP9), stromelysin-specific (eg, MMP3), and membranous types (eg, MT1-MMP). MMPs have multiple roles in the events that underlie ventricular remodeling after infarction and development of ventricular failure in the heart. For instance, mice deficient for MMP9, a gelatinase, have reduced remodeling after infarct and improved function, suggesting that MMP9 activity contributes to fibrosis.²² On the other hand, MMPs with collagenase activity are anti-fibrotic.²³ Dysregulation of MMP activity is generally associated with adverse myocardial remodeling.^{23,24} Thus, zinc potentially plays multiple regulatory roles relating to collagen deposition and degradation. The article by Wang and colleagues³ in this issue of *The American Journal of Pathology* confirms this by demonstrating that zinc deficiency alters the cardiomyopathic response to alcohol in the mouse.

Animal Models of ACM

Curiously, although alcohol induces hypertrophy and cardiac dysfunction in mice, fibrosis is not typically part of the pathophysiological picture. Thus, murine models of ACM have been problematic and of dubious clinical application. Investigators have turned to other animals, including the dog, rabbit, rat, and chicken,^{7,25–35} to develop more relevant models. Still, the features of ACM in these animal models do not exactly mirror those of human ACM and are completely reversible by withdrawal of

alcohol, unlike the human condition.⁷ Nevertheless, use of these models has enabled a great deal of research and has revealed several important aspects of alcohol toxicity in the cardiovascular system. Although animal models have their limitations, many of the experimental approaches that have been used with these models could not be performed with humans due to obvious ethical dilemmas. However, the paucity of murine models in particular poses a severe limitation because one cannot take advantage of the powerful approaches available, including genetically engineered mice (transgenics and gene targeting), the mouse genome, gene expression and proteomic databases, the numerous inbred, outbred, mutant strains, and the inbred recombinant and congenic lines that have been engineered. Currently, many such resources for the mouse exist and their number and availability increase almost daily. Mouse models of cardiovascular disease have proven to be extremely powerful tools for the elucidation of pathophysiological mechanisms. Thus, developing mouse models of ACM that more closely mirror the human condition is paramount.

The Murine MT-KO Model of ACM

In their study, Wang and colleagues³ demonstrate that mice homozygous for a deficiency of both the MT I and II genes (MT-KO) present with an ACM more typical of the human disease because fibrosis occurs. In this model, alcohol feeding was accomplished using a Lieber and DeCarli liquid diet modified by replacing the alcoholic diet with the control diet on the last day of each week; this prevented the weight loss that otherwise is associated with long-term alcohol feeding regimens of this type. The content of alcohol was increased during the 2-month regimen, from 26 to 36% of total calories. Importantly, the blood alcohol levels in this model are close to those measured in alcoholic patients.³ At the 2-month time point, wild-type and MT-KO mice were euthanized, and the effects of alcohol on the morphological and histological features of cardiomyopathy were ascertained. Analysis of areas of collagen accumulation showed a significant twofold increase in fibrosis in MT-KO relative to wild-type mice treated with alcohol. This reflects increases in both perivascular and interstitial fibrosis. In MT-KO mice, this was associated with neutrophil infiltration and cardiomyocyte hypertrophy in regions of focal fibrotic lesions. This likely represents replacement fibrosis, which is quite characteristic of cardiomyopathy, including human ACM. This is the first important aspect of the article by Wang and colleagues,³ the development of the first mouse ACM model that recapitulates fibrosis, a critical aspect of pathophysiology associated with human ACM.

The second important aspect of this work is that fibrosis in the MT-KO/ACM model is reversible by zinc.³ The investigators used zinc supplementation with 100 mg of zinc per L liquid diet and found this sufficient to prevent the alcohol-induced decrease of zinc in the livers of experimental animals. In the MT-KO/ACM model, zinc supplementation significantly reduced fibrosis overall

Figure 1. Conceptualization of the effect of zinc and MT on cardiac fibrosis in MT-KO mice. Multiple anti-fibrotic and profibrotic factors (right and left, respectively) contribute to the homeostatic balance that characterizes normal physiology. These are imbalanced in the heart by chronic alcohol exposure. Ethanol exposure increases catecholamine synthesis and oxidative stress, reduces myocardial zinc content, and indirectly activates inflammatory signaling, leading to increased cytokine levels. In the human, this is sufficient to result in cardiomyopathy associated with irreversible tissue remodeling including fibrosis. In the mouse, however, further perturbation of zinc concentration, via abrogation of endogenous MT, is required to tip the balance far enough to result in fibrosis. Dietary supplementation with zinc can oppose these changes and significantly reduce fibrosis in the MT-KO/ACM model.³ **Light arrows** are anti-fibrotic effects while **dark arrows** are profibrotic effects.

and nearly prevented interstitial fibrosis. The zinc supplementation did not affect the alcohol-induced cardiac hypertrophy measured by heart-to-body weight ratio. Importantly, hypertrophy was similar in wild-type and MT-KO mice subjected to the alcohol diet for 2 months. Zinc supplementation also suppressed some of the alcohol-induced mitochondrial dysmorphogenesis and was more effective in this respect in wild-type relative to MT-KO mice. In addition to delineating a role in fibrosis, these results suggest that zinc and MT may play important anti-oxidant roles in this model.

The seminal discovery by Wang and colleagues,³ that alcohol-induced fibrosis occurs in mice deficient for MT and is suppressed by zinc supplementation, demonstrates that zinc homeostasis is critical for development of fibrosis in murine ACM. Although reduced tissue zinc levels were previously associated with alcoholic pathophysiology in the heart and liver, the causal relationship was unknown. Fibrosis has been well studied in multiple organs and appears to be a critical component of wound healing after injury. As such, fibrosis has multiple critical components and is intimately linked to inflammatory signaling and cell-cell interactions between tissue-resident cells and infiltrating cells.³⁶ In the heart, fibrosis is associated with increased proliferation of fibroblasts, increased transdifferentiation of fibroblasts to myofibroblasts, and increased deposition of collagen.³⁷ In ACM, ethanol exposure tips the normal homeostatic balance between anti- and profibrotic signals in the direction of fibrosis (Figure 1). Critical alcohol-dependent profibrotic signals include increased catecholamine levels, oxidative stress, inflammatory cytokine levels, and as shown by Wang and colleagues,³ reduced zinc levels.^{6–8}

Reduction of zinc and MT act in multiple ways to exacerbate fibrosis. As discussed above, zinc may play a critical role in regulating the activities of proteases involved in extracellular matrix remodeling and may also be necessary for the activity of anti-oxidant proteins. MT has been shown to be critically involved in zinc homeostasis³⁸ and to release zinc subsequent to oxidative stress.^{39,40} MT may therefore act as a sink for zinc, in which case its absence contributes to decreased tissue zinc levels and exacerbation of oxidative stress after ischemia or alcohol exposure. This is supported by the fact that MT and zinc levels are decreased coordinately in tissues of humans and animals chronically exposed to alcohol and the observation that zinc levels are reduced in the livers of MT-KO mice.^{3,8,18} MT is known to have reactive oxygen species scavenging capability^{17,41} and may also act directly as an reactive oxygen species scavenger to reduce the oxidative effects of alcohol. Thus, after ethanol exposure, low zinc levels and MT deficiency would tend to tip the homeostatic balance that exists in the normal myocardium further toward fibrosis (Figure 1). One would therefore predict that zinc supplementation would at least partially restore the decreased pool of zinc and improve the function of MT and collagenase MMPs, thereby reducing fibrosis (Figure 1). If zinc works partially through MT, this effect would be predicted to be stronger in wild-type than in MT-KO mice, exactly as observed by Wang and colleagues.³

Summary

The study by Wang and colleagues³ in this issue of *The American Journal of Pathology* describes a novel murine model of ACM. Results from experiments that use this model support the hypothesis that zinc availability critically affects fibrosis in ACM *in vivo*. In humans, chronic alcohol abuse is associated with irreversible fibrosis and dilated cardiomyopathy. In animal models, however, fibrosis can be reversed by removing alcohol from the diet or, as in this study, by zinc supplementation. As discussed above, zinc supplementation has been previously reported to reverse alcoholic liver fibrosis in the carbon tetrachloride liver injury model.¹⁵ It is currently unknown whether the reversibility of fibrosis and other features of ACM in animals, whether by alcohol withdrawal or zinc supplementation, is related to differences in basic mechanisms or to the fact that human disease is most often associated with much longer duration of alcohol usage.⁷ Despite this conundrum, studying the mechanisms that underlie the effects of alcohol on cardiac function and remodeling will likely result in significant new knowledge that is clinically relevant. Whether it proves efficacious to completely block the fibrotic component of remodeling remains to be seen; certain aspects of fibrosis and wound healing are likely required for the heart to sustain functional integrity in certain circumstances. Nevertheless, understanding the mechanisms that underlie reversal of fibrosis is of great interest. The existence of the MT-KO/ACM model will allow investigators to bring the power of mouse genetics to bear on the problems of

ACM and cardiac fibrosis, increasing the probability that detailed aspects of the molecular mechanism will be forthcoming. These results will eventually have great impact in identifying novel therapeutic targets for limiting pathological fibrosis in the clinic.

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References

- Piano MR: Alcoholic cardiomyopathy incidence, clinical characteristics, and pathophysiology. *Chest* 2002, 121:1638–1650
- See F, Kompa A, Martin J, Lewis DA, Krum H: Fibrosis as a therapeutic target post-myocardial infarction. *Curr Pharm Des* 2005, 11:477–487
- Wang L, Zhou Z, Saari JT, Kang YJ: Alcohol-induced myocardial fibrosis in metallothionein-null mice: prevention by zinc supplementation. *Am J Pathol* 2005, 167:337–344.
- Zhang X, Lia S-Y, Brown RA, Ren J: Ethanol and acetaldehyde in alcoholic cardiomyopathy: from bad to ugly en route to oxidative stress. *Alcohol* 2004, 32:175–186
- Thomas AP, Rozanski DJ, Renard DC, Rubin E: Effects of ethanol on the contractile function of the heart: a review. *Alcohol Clin Exp Res* 1994, 18:121–131
- Vendemiale G, Grattagliano I, Altomare E, Serviddio G, Portincasa P, Prigigallo F, Palasciano G: Mitochondrial oxidative damage and myocardial fibrosis in rats chronically intoxicated with moderate doses of ethanol. *Toxicol Lett* 2001, 123:209–216
- Ponnappa BC, Rubin E: Modeling alcohol's effects on organs in animal models. *Alcohol Res Health* 2000, 24:93–104
- Rodriguez-Moreno F, Gonzalez-Reimers E, Santolaria-Fernandez F, Galindo-Martin L, Hernandez-Torres O, Batista-Lopez N, Molina-Perez M: Zinc, copper, manganese, and iron in chronic alcoholic liver disease. *Alcohol* 1997, 14:39–44
- Bogden JD, Al-Rabiai S, Gilani SH: Effect of chronic ethanol ingestion on the metabolism of copper, iron, manganese, selenium, and zinc in an animal model of alcoholic cardiomyopathy. *J Toxicol Environ Health* 1984, 14:407–417
- Topuzoglu G, Erbay AR, Karul AB, Yensel N: Concentrations of copper, zinc, and magnesium in sera from patients with idiopathic dilated cardiomyopathy. *Biol Trace Elem Res* 2003, 95:11–17
- Oster O: Trace element concentrations (Cu, Zn, Fe) in sera from patients with dilated cardiomyopathy. *Clin Chim Acta* 1993, 214:209–218
- Mills PR, Fell GS, Bessent RG, Nelson LM, Russell RA: A study of zinc metabolism in alcoholic cirrhosis. *Clin Sci* 1983, 64:527–535
- Milman N, Laursen J, Podenphant J, Asnaes S: Trace elements in normal and cirrhotic human liver tissue I. Iron, copper, zinc, selenium, manganese, titanium and lead measured by X-ray fluorescence spectrometry. *Liver* 1984, 6:111–117
- Valberg LS, Flanagan PR, Ghent CN, Chamberlain MJ: Zinc absorption and leukocyte zinc in alcoholic and nonalcoholic cirrhosis. *Dig Dis Sci* 30:329–333
- Anttinen H, Ryhanen L, Puistola U, Arranto A, Oikarinen A: Decrease in liver collagen accumulation in carbon tetrachloride-injured and normal growing rats upon administration of zinc. *Gastroenterology* 1984, 86:532–539
- Gimenez A, Pares A, Alie S, Camps J, Deulofeu R, Caballeria J, Rodes J: Fibrogenic and collagenolytic activity in carbon-tetrachloride-injured rats: beneficial effects of zinc administration. *J Hepatol* 1994, 21:292–298
- Cabre M, Camps J, Ferre N, Paternain JL, Joven J: The antioxidant and hepatoprotective effects of zinc are related to hepatic cytochrome P450 depression and metallothionein induction in rats with experimental cirrhosis. *Int J Vitam Nutr Res* 2001, 71:229–236
- Elsheirif L, Jiang Y, Saari JT, Kang YJ: Dietary copper restriction-induced changes in myocardial gene expression and the effect of copper repletion. *Exp Biol Med (Maywood)* 2004, 229:616–622
- Rojkind M, Dunn MA: Hepatic fibrosis. *Gastroenterology* 1979, 76:849–863
- Camps J, Bargallo T, Gimenez A, Alie S, Caballeria J, Pares A, Joven J, Masana L, Rodes J: Relationship between hepatic lipid peroxidation and fibrogenesis in carbon tetrachloride-treated rats: effect of zinc administration. *Clin Sci (Lond)* 1992, 83:695–700
- Otsu K, Ikeda Y, Fujii J: Accumulation of manganese superoxide dismutase under metal-depleted conditions: proposed role for zinc ions in cellular redox balance. *Biochem J* 2004, 377:241–248
- Ducharme A, Frantz S, Aikawa M, Rabkin E, Lindsey M, Rohde LE, Schoen FJ, Kelly RA, Werb Z, Libby P, Lee RT: Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. *J Clin Invest* 2000, 106:55–62
- Lindsey ML: MMP induction and inhibition in myocardial infarction. *Heart Fail Rev* 2004, 9:7–19
- Tsuruda T, Costello-Boerrigter LC, Burnett Jr JC: Matrix metalloproteinases: pathways of induction by bioactive molecules. *Heart Fail Rev* 2004, 9:53–61
- Chan TC, Sutter MC: The effects of chronic ethanol consumption on cardiac function in rats. *Can J Physiol Pharmacol* 1982, 60:777–782
- Regan TJ, Morvai V: Experimental models for studying the effects of ethanol on the myocardium. *Acta Med Scand Suppl* 1987, 717:107–113
- Capasso JM, Li P, Guideri G, Malhotra A, Cortese R, Anversa P: Myocardial mechanical, biochemical, and structural alterations induced by chronic ethanol ingestion in rats. *Circ Res* 1992, 71:346–356
- Ma Z, Miyamoto A, Lee SS: Role of altered beta-adrenoceptor signal transduction in the pathogenesis of cirrhotic cardiomyopathy in rats. *Gastroenterology* 1996, 110:1191–1198
- Richardson PJ, Patel VB, Preedy VR: Alcohol and the myocardium. *Novartis Found Symp* 1998, 216:35–45
- Liang Q, Carlson EC, Borgerding AJ, Epstein PN: A transgenic model of acetaldehyde overproduction accelerates alcohol cardiomyopathy. *J Pharmacol Exp Ther* 1999, 291:766–772
- Morris N, Kim CS, Doye AA, Hajjar RJ, Laste N, Gwathmey JK: A pilot study of a new chicken model of alcohol-induced cardiomyopathy. *Alcohol Clin Exp Res* 1999, 23:1668–1672
- Tabakoff B, Hoffman PL: Animal models in alcohol research. *Alcohol Res Health* 2000, 24:77–84
- Kim SD, Beck J, Bieniarz T, Schumacher A, Piano MR: A rodent model of alcoholic heart muscle disease and its evaluation by echocardiography. *Alcohol Clin Exp Res* 2001, 25:457–463
- Ward CA, Liu H, Lee SS: Altered cellular calcium regulatory systems in a rat model of cirrhotic cardiomyopathy. *Gastroenterology* 2001, 121:1209–1218
- Kim SD, Bieniarz T, Esser KA, Piano MR: Cardiac structure and function after short-term ethanol consumption in rats. *Alcohol* 2003, 29:21–29
- Azouz A, Razzaque MS, El-Hallak M, Taguchi T: Immunoinflammatory responses and fibrogenesis. *Med Electron Microsc* 2004, 37:141–148
- Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA: Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenyl cyclase. *Proc Natl Acad Sci USA* 2005, 102:437–442
- Maret W: The function of zinc metallothionein: a link between cellular zinc and redox state. *J Nutr* 2000, 130(5S Suppl):1455S–1458S
- Vallee BL: The function of metallothionein. *Neurochem Int* 1995, 27:23–33
- Maret W, Vallee BL: Thiolate ligands in metallothionein confer redox activity on zinc clusters. *Proc Natl Acad Sci USA* 1998, 95:3478–3482
- Gobel H, van der Wal AC, Teeling P, van der Loos CM, Becker AE: Metallothionein in human atherosclerotic lesions: a scavenger mechanism for reactive oxygen species in the plaque? *Virchows Arch* 2000, 437:528–533