could have been generated during the chloroform-phenol extraction because the internal standard was not added until after the enzymatic hydrolysis and the use of phenol for DNA extraction has been associated with artefactual generation of 8-hydroxy-2'-deoxyguanosine. Furthermore, some readers might object to the use of ascorbic acid as an antioxidant because iron-ascorbate systems have often been used experimentally to promote hepatic lipid peroxidation. However, in answer to the second question raised, about the ability of lipid peroxides to promote oxidative DNA damage *in vivo*, there can be little doubt that the authors have succeeded in demonstrating that malondialdehyde does form adducts with DNA in human liver.

However, one can and should question the conclusion drawn by the authors that M_1G can be detected in healthy individuals with "disease-free" livers in amounts "comparable to the highest levels of adducts reported in tissues from individuals exposed to exogenous carcinogens." The major reason for raising this question is the lack of information about both the organ donors themselves as well as the handling of the samples.

It is stated that the livers were obtained from the Tennessee Donor Services and stored at −80°C. Given the extreme scarcity of donor livers in the United States at the present time and the fact that, at the very least, the livers would have been offered to critically ill status IV recipients on a nationwide basis, the ability to use gram quantities of the livers for research purposes suggests that for some unknown reason, the six livers were not judged suitable for transplantation into any recipient. For example, one might surmise that the 185 pound female and the 232 pound male had fatty livers. Three of the six donors died of motor vehicle accidents and the alcohol history is not stated.

Other unknown factors that might promote oxidative damage in the liver samples include possible ischemia/reperfusion injury before harvesting, duration of time between harvest and storage at −80°C, as well as storage temperatures during this interval, and type of preservation solution. Although these concerns do not invalidate the careful techniques used by the authors, it would be desirable to have as much information as possible before accepting the quantitation of the adducts as representative of *in vivo* processes in healthy individuals. In other words, the same scientific rigor that was applied to the development of the analytical methods should also be applied to the source and description of the human liver samples. Thus, much work remains to find complete answers to the third question, relating to quantification of oxidative DNA damage in normal livers, as well as to the fourth question, pertaining to the quantitative threshold of oxidative DNA damage and genotoxicity.

However, these questions are deserving of careful responses, because of the potential relevance of these questions to hepatocarcinogenicity in humans. Ascertainment of a link between free radical damage to DNA and hepatocellular carcinoma would suggest that prevention might be possible with judicious chronic administration of antioxidants. If it becomes possible to detect M_1G in urine as has been possible with other markers of DNA damage,[10] then this adduct may become a useful dosimeter of this type of damage to human liver DNA. If monoclonal antibodies can be raised against this adduct, then it should become possible to identify it in percutaneous liver biopsy samples, thereby avoiding the potential pitfalls and practical difficulties involved in the gram quantities of fresh tissue needed at the present time for the analysis. Chaudhary et al should be congratulated on paving the way to these possibilities.

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MOLECULAR MECHANISM OF HEPATOCELLULAR INJURY IN ALPHA₁ ANTITRYPSIN DEFICIENCY

Wu Y, Whitman I, Molmenti E, Moore K, Hippenmeyer P, Perlmutter DH. A lag in intracellular degradation of mutant alpha₁-antitrypsin correlates with the liver disease phenotype in homozygous PiZZ alpha₁-antitrypsin deficiency. Proc Natl Acad Sci U S A 1994;91:9014-9018.

ABSTRACT

Liver injury in PiZZ α_1-antitrypsin (α_1-AT) deficiency probably results from toxic effects of the abnormal α_1-AT molecule accumulating within the ER of liver cells.

However, only 12-15% of individuals with this same genotype develops liver disease. Therefore, we predicted that other genetic traits that determine the net intracellular accumulation of the mutant α_1-AT molecule would also determine susceptibility to liver disease. To address this prediction, we transduced skin fibroblasts from PiZZ individuals with liver disease or without liver disease with amphotropic recombinant retroviral particles designed for constitutive expression of the mutant α_1-AT Z gene. Human skin fibroblasts do not express the endogenous α_1-AT gene but presumably express other genes involved in postsynthetic processing of secretory proteins. The results show that expression of human α_1-AT gene was conferred on each fibroblast cell line. Compared to the same cell line transduced with the wild-type α_1-AT M gene, there was selective intracellular accumulation of the mutant α_1-AT Z protein in each case. However, there was a marked delay in degradation of the mutant α_1-AT Z protein after it accumulated in the fibroblasts from ZZ individuals with liver disease ("susceptible hosts") as compared to those without liver disease ("protected hosts"). Appropriate disease controls showed that the lag in degradation in susceptible hosts is specific for the combination of PiZZ phenotype and liver disease. Biochemical characteristics of α_1-AT Z degradation in the protected hosts were found to be similar to those of a common ER degradation pathway previously described in model experimental cell systems for T-cell receptor α subunits and asialoglycoprotein receptor subunits, therefore, raising the possibility that the lag in degradation in the susceptible host is a defect in this common ER degradation pathway. Thus, these data provide evidence that other genetic traits that affect the fate of the abnormal α_1-AT Z molecule, at least in part, determine susceptibility to liver disease. These data also validate a system for elucidating the biochemical/genetic characteristics of these traits and for examining the relevance to human disease of pathways for protein degradation in the ER.

COMMENTS

The common genetic disease alpha$_1$ antitrypsin (alpha$_1$ AT) deficiency is associated with liver disease in 12% to 15% of patients who are homozygous for the Z variant–deficient phenotype (PiZZ).[1] Alpha$_1$ AT is the major circulating serine protease inhibitor whose anti-elastase activity prevents neutrophil elastase from destroying the lung. The protein is made in the liver and its deficiency results in plasma concentrations of secreted functional protein that are 10% to 15% of normal. There are variable mutations leading to the deficient phenotype with a gene frequency of 1 in 40 alleles in Caucasian patients. The most common Z deficiency variant studied in the article by Wu et al is a single nucleotide Glu 342 → Lys substitution in the alpha$_1$ AT coding sequence. The homozygous PiZZ alpha$_1$ AT phenotype occurs in 1 in 1,600 live births.[2] The mechanism by which 1 of every 12,000 people develops liver disease from alpha$_1$ AT deficiency has been a focus of research in molecular hepatology and a matter of some speculation.

The most common pathology from alpha$_1$ AT deficiency involves elastolytic attack on the lung as a result of lowered levels of the anti-elastase alpha$_1$ AT. This pathology is exacerbated by additional pulmonary insults, such as cigarette smoke, toxins, or infections. Therapeutic strategies have involved avoidance of pulmonary insults or, ultimately, lung transplantation. Experimental gene therapy approaches to the pulmonary pathology involve expression of normal alpha$_1$ AT in the liver with its subsequent circulation to the lung.[3]

Therapeutic approaches to prevent alpha$_1$ AT hepatic pathology have been stymied by a less than complete understanding of why only a fraction of patients with alpha$_1$ AT develop liver disease and of the mechanism of hepatic injury. Several lines of evidence suggest that accumulation of the mutant protein in hepatocytes is one important factor leading to hepatic pathology. The mutant protein folds differently than the wild-type protein and is caught up in the endoplasmic reticulum (ER) and poorly secreted.[4] In transgenic animal studies, expression of the mutant protein led to hepatic pathology.[5,6] These animals had normal murine alpha$_1$ AT, therefore, it was the presence of the mutant protein rather than the absence of anti-elastolytic activity that resulted in hepatic pathology.

It was in this context that Wu et al postulated that alpha$_1$ AT patients with liver disease processed the mutant protein differently than patients who expressed the same mutant protein who do not develop liver disease. To study this process in more detail, primary fibroblasts derived from patients were used to compare the posttranslational pathways of those with and without liver disease. Fibroblasts do not normally express alpha$_1$ AT, but the assumption is that their protein secretory and degradation pathways are comparable with that of hepatocytes.

Retroviral vectors were constructed to express wild-type or Z variant alpha$_1$ AT. These vectors were then used to produce stably transduced cell lines of fibroblasts expressing wild-type or mutant alpha$_1$ AT. Human fibroblasts were derived using punch biopsy from three groups of patients: (1) those with liver disease and alpha$_1$ AT deficiency, (2) those with alpha$_1$ AT deficiency but no liver disease, and (3) those with liver disease and wild-type alpha$_1$ AT. These fibroblasts were then transduced with the alpha$_1$ AT retroviral expression vectors and analyzed for synthesis, kinetics of secretion, and glycosylation of wild-type and Z variant alpha$_1$ AT.

Transfected cells were pulse labeled with ^{35}S methionine, and after immunoprecipitation sodium dodecylsulfate–polyacrylamide gel electrophoresis fluorography showed production of the 52-kd coded protein and 55-kd posttranslationally modified glycopeptide. Next the kinetics of conversion of the 52-kd protein to the 55-kd protein by glycosylation and subsequent secretion were examined by pulse chase immunoprecipitation analysis of cell lysate and extracellular media. The cell lysate showed intracellular accumulation of the glycosylated mutant Z protein while the wild-type M protein was secreted into extracellular media. The glycosylated protein converts from endoglycosidase H

(endo-H) sensitive to endo-H resistant in the ER immediately before secretion, and the mutant protein was shown to remain in the endo-H sensitive state, suggesting accumulation in the ER.

Immunofluorescent studies were performed to localize the cellular compartment of mutant protein accumulation and Z protein stained in a reticular cytoplasmic pattern strongest around the nucleus. Further immunofluorescence studies showed this pattern to colocalize with BiP, a well-defined ER protein. The endo-H and immunofluorescence studies show that retrovirally expressed Z protein is immaturely glycosylated and accumulates in the ER of fibroblasts, just as in the disease state in *in vivo* hepatocytes.

The kinetics of mutant protein accumulation were compared in fibroblasts from patients with normal alpha$_1$ AT and liver disease, alpha$_1$ AT deficiency without liver disease, and alpha$_1$ AT with liver disease. After transduction with the Z protein expression vector, the fibroblasts from the ZZ patient susceptible to liver disease showed accumulation of the abnormal Z alpha$_1$ AT molecule. In contrast, the ZZ patient without liver disease and the patient with severe liver disease and MM phenotype showed no accumulation of alpha$_1$ AT after transduction. These results in concert with controls from several other patients suggest that accumulation of alpha$_1$ AT is responsible for liver injury. Liver injury results from the concordant existence of the expression of the abnormally folded Z alpha$_1$ AT protein and a delay in the ER protein degratory pathway resulting in accumulation of the Z protein. In accordance with this hypothesis, patients who produce Z protein but are able to clear the abnormal protein will not develop liver disease. Only those patients who both produce abnormal protein and have a relative delay in transduction of the abnormal protein should develop liver disease.

Another line of evidence supporting this hypothesis is the observation that MZ heterozygotes with liver disease often have additional viral or alcohol insults, which may predispose to either increased production of alpha$_1$ AT or an additional lag in its degradation.

The article by Wu et al validates a primary fibroblast system to study the kinetics of mutant protein degradation and to correlate it with liver disease phenotype. Such a system might be used to screen individuals with ZZ alpha$_1$ AT for susceptibility to liver disease.

In summary, the article by Wu et al lends further support to the hypothesis that liver disease in alpha$_1$ AT deficiency results from accumulation of mutant protein in hepatocytes. The concordant production of mutant protein and a lag in degradation of the misfolded Z protein lead to the liver disease phenotype in a subset of patients with alpha$_1$ AT deficiency. Only a small percentage of patients with alpha$_1$ AT deficiency develop liver disease because they must not only produce the mutant protein but also have a defect in their ER major degratory pathway for misfolded proteins.

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