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SAMe Treatment Prevents the Ethanol-Induced Epigenetic Alterations of Genes in the Toll-Like Receptor Pathway

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Abstract

Prior studies showed that Toll-like receptor (TLR) signaling pathway genes were up regulated in the liver of rats fed ethanol, but not in rats fed ethanol plus S-adenosylmethionine (SAME). These results were obtained using a PCR microplate array analysis for TLRs and associated proteins such as proinflammatory cytokines and chemokine mRNA levels. A large number of genes were up regulated by the ethanol diet, but not the ethanol plus SAME diet. In the present study, using the same experimental rat livers, DNA methylation analysis was done by using an Epiect Methyl DNA Restriction Kit (Qiagen, 335451) (24 genes). The results of all the genes combined shows a highly significant increase in methylation in the ethanol-fed group of rats, but not in the dextrose-fed, SAME-fed or ethanol plus SAME-fed groups of rats. There was also an increase in DNA methylation in rats with high blood alcohol levels compared to a rat with a low blood alcohol level. The individual genes that were up regulated as indicated by the increased mRNA measured

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by qPCR correlated positively with the increased methylation of the DNA of the corresponding gene as follows: Cd14, Hspa1a, Irf1, Irak1, Irak2, Map3k7, Myd88, Ppara, Ripk2, Tollip and Traf6.

Keywords

TLR (Toll-like receptor); SAME (S-adenosyl methionine); BAL/blood alcohol levels; 5-methylcytosine

Introduction

The TLR signaling pathway activation is involved in chronic liver injury and liver carcinogenesis (Bardag-Gorce et al., 2010b; Broering et al., 2011; Machida et al., 2012; Oliva et al., 2011b; Oliva and French, 2012). The up regulation of the TLR4 signaling pathway is prevented by feeding S-adenosylmethionine (SAME) suggesting that DNA methylation of the genes in the pathway is involved in its regulation (Bardag-Gorce et al., 2010b; Oliva et al., 2011a; Oliva and French, 2012). This was true when ethanol was fed to rats for one month (Bardag-Gorce et al., 2010a). To further explore this phenomenon we measured the methylation of DNA of 24 genes in the TLR4 pathway using the Methyl Signature qPCR Array kit. Although the study of methylation of the individual 24 genes showed only a trend in an increase in DNA methylation in the livers of the ethanol fed animals, when the results of all 24 genes were combined, the increase in methylation was highly significant, and this up regulation was completely prevented by feeding SAME with ethanol.

Methods

Animals

The data is from the livers of 200g Male Wistar rats (Bardag-Gorce et al., 2010a). The rats were fed ethanol with or without SAME supplement for 1 month. The results of the morphology studies and studies of the TLR signaling pathway from these livers have been reported previously (Bardag-Gorce et al., 2010a; Oliva et al., 2011b). A portion of the fast frozen livers stored at -80°C were used in the present study to further characterize the effects of ethanol and SAME on the expression of cytokines and the TLR pathway using methylation qPCR (n=3 per 4 groups i.e. Dextrose control; SAME control; ethanol; and ethanol plus SAME). The ethanol-fed rats were sacrificed at high blood alcohol level (BAL), whereas, the ethanol plus SAME (~500 g)-fed rats were sacrificed at ~200 g BAL to determine the effects of BAL on DNA methylation.

Immunohistochemistry

Rat livers were immunostained with an antibody to 5-methylcytosine (Calbiochem, NA81) to view the intensity of hepatocytic nuclear DNA global methylation.

Tissue DNA extraction

Total DNA was extracted from rat liver sections by using DNeasy Blood and Tissue Kit (Qiagen, 69504) according to the manufacturer's instructions.

Restriction digestion of tissue DNA

The tissue DNA extracts were digested in preparation for methylation analysis by using Epitect Methyl DNA Restriction Kit (Qiagen, 335451) according to the manufacturer's instructions.

Methylation qPCR

The digested DNA samples were analyzed for methylation of the components of the Toll-like receptor signaling pathway by using EpiTect Methyl Signature qPCR Array (Qiagen, 335211 MeAR-181A) according to the manufacturer's instructions.

DNA methylation analysis

DNA methylation levels were determined by the EpiTect Methyl DNA Methylation PCR Data Analysis software template for the MeAR-181A kit (Qiagen, http://www.sabiosciences.com/dna_methylation_data_analysis.php) according to the software instructions.

Statistical analysis

Error bars reflect standard error of the mean. P values were determined by student t test.

Results

Hepatocyte nuclei show variation in methylation

The immunostain for 5-methylcytosine showed variation in fluorescence intensity of neighboring liver cell nuclei in the ethanol fed rats but not in the control rats (Fig. 1).

Methylation profile of the components of the TLR signaling pathway

We determined the methylation levels of 24 genes involved in the Toll-like signaling pathway (Fig. 2). Total DNA was extracted from rat livers, restriction digested in preparation for methylation analysis, and subjected to methylation quantitative polymerase chain reaction (qPCR) analysis. The raw values obtained by the methylation analysis software indicate a trend in increased methylation levels of the majority of genes in ethanol-fed rats, which was prevented by SAME feeding (Fig. 2). The changes seen in individual genes were not statistically significant, although there appeared to be a trend towards increased methylation in the presence of ethanol. The large amount of deviation within samples may be secondary to variation in methylation of hepatocyte nuclei as well as contamination from other cell types.

SAME treatment prevented the ethanol-induced increase in total methylation levels of the TLR signaling pathway genes

Fig. 2 represents the methylation levels in the nuclei of all cell types found in liver tissue samples including hepatocytes, Kupffer cells, endothelium and bile duct cells. As shown by immunofluorescence staining, variation between hepatocyte nuclei occurred in the total levels of DNA methylation of hepatocytes (Fig. 1). Alterations in methylation levels of individual genes in hepatocytes alone may not be well-resolved in the analysis of methylation levels of total tissue DNA. Consequently, we measured the average methylation levels of all the 24 genes in the TLR signaling pathway for the four experimental groups. As shown in Fig. 3, ethanol treatment significantly increased the average methylation levels of the TLR signaling pathway genes compared with the control dextrose treatment. Furthermore, SAME treatment completely prevented the ethanol-induced increase in methylation levels to the same level as in dextrose controls, while no significant changes were observed for the SAME plus dextrose treatment when compared with dextrose treatment alone (Fig. 3).

Ethanol treatment increased the total methylation levels of the TLR signaling pathway in a dose dependent manner

For each of the three ethanol-fed rats, we measured the average methylation levels of all 24 genes in the TLR signaling pathway. Rat 44 had a low blood alcohol level at the time of sacrifice, while rats 45 and 46 had much higher blood alcohol levels. As shown in Fig. 4, the total methylation levels of the TLR signaling pathway significantly increased with higher blood alcohol levels.

Discussion

It was not anticipated that ethanol feeding would increase the expression of the TLR signal pathway genes by increasing DNA methylation because SAdMe, a methyl donor, prevented this effect of ethanol feeding. However, when we correlated the changes in methylation of the individual genes with the gene expression changes, we found that when the gene expression increased, the level of DNA methylation tended to increase. For instance; the previous studies of gene expression qPCR microarray data (Oliva et al., 2011b) correlated positively with the DNA methylation data (Fig 2) as follows: Cd14, Hspa1a, Irf1, Irak1, Irak2, Map3KU7, MyD88, Ppara, Ripk2, Tollip and Traf6. Likewise, protein levels of MyD88 correlated with the DNA methylation data (Fig 2).

When all of the DNA methylation data was combined from the 24 genes from the 4 groups of 3 rats each, the increase in DNA methylation by feeding alcohol was highly significant when compared with the 3 other groups (Fig 3). The 3 other groups including the ethanol plus SAdMe group did not differ from each other. This indicates that SAdMe prevents the increase in the DNA methylation caused by ethanol (Fig 3). Additionally, the increase in BAL also increased the level of DNA methylation compared to a low level of BAL.

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List of abbreviations

DEX	dextrose
EtOH	ethanol
qPCR	quantitative polymerase chain reaction
SAdMe	S-adenosylmethionine
TLR	Toll-like receptor

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Fig. 1.

Control liver (A) and ethanol fed rat liver (B) were stained with an antibody to 5-methylcytosine. The intensity of the stain was recorded morphometrically and viewed as screen hunter. Note the intensity peaks of the liver nuclei vary when compared to each other in the ethanol fed rat (B). The nuclei of the control rat do not vary in intensity (A). (intensity measured at 167 for A and B).

Fig. 2.

Percentage of methylated DNA of 24 genes involved in the Toll-like receptor signaling pathways is shown. Total DNA was extracted from rat liver sections and subjected to restriction digestion in preparation for methylation analysis. Methylation qPCR was performed on the digested DNA samples and the percentage of methylated DNA was determined. Error bars represent the Mean $\pm$ SEM (n=3).

Fig. 3. SAmE treatment prevents the ethanol-induced increase in total methylation levels of the genes in the TLR signaling pathway. The average methylation levels of the TLR signaling pathway for each of the four experimental groups was calculated. Error bars represent the Mean $\pm$ SEM (n=3).

Fig. 4. Ethanol treatment increases the total methylation levels of the TLR signaling pathways when comparing the effect of a high BAL with a low BAL. The average levels of methylation of the TLR signaling pathways correlate with the ethanol doses. Error bars represent the Mean $\pm$ SEM.