Effects of Chronic Alcohol Use on Mouse Myocardial Tissue

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Summary

Alcohol intake induces cardiovascular effects. We performed a comprehensive analysis of the gene expression profiles of myocardial tissues from a mouse model of alcohol feeding by microarray to clarify the effect of alcohol on myocardium from the perspective that the acute phase of alcohol use causes changes to myocardial injury related gene expression profiles, while the withdrawal phase is associated with changes in gene expression profiles related to myocardial protection. A model of single alcohol feeding was generated using pathogen-free, 7-week-old, male C57BL/6N mice administered a peroral injection of 7.0 g/kg ethanol (single alcohol-fed group group) or saline (control group). A repeated alcohol feeding group was generated following Lieber's method. To investigate the effects of alcohol in the alcohol-fed group, the left ventricles of hearts were extirpated 1 h (alcohol group) or 24 h (withdrawal group) after the last alcohol feeding. Myocardial tissues were then subjected to RNA extraction and gene expression analysis by real-time reverse transcription polymerase chain reaction. Sera were also analyzed for various signal transducers, including 22 cytokines. Using RNA extracted from extirpated myocardia, mRNA expressions were comprehensively analyzed by one-color microarray (n=4) using SurePrint G3 Mouse Gene Expression 8x60K (Agilent) containing 38,640 gene probes as the microchip, and gene ontology and gene cascade analyses were performed. Differing gene profiles were seen in the repeated alcohol intake group. In the acute phase of alcohol intake, there were changes in gene profiles related to activation of the STAT pathway and blood cytokine-mediated inflammatory responses to hypercardia. In the withdrawal from alcohol phase, changes in gene profiles related to STAT activity did not persist, although gene

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profile changes related to apoptosis and the development of sudden death were observed. Further detailed investigations of the effects of alcohol on cardiomyocytes are planned.

Key words: alcoholism, sudden death, cardiac hypertrophy, microarray, mice

Introduction

Alcohol is metabolized to acetic acid via acetaldehyde metabolism, mainly in the liver[1]. However, if the liver undergoes excessive metabolism, metabolites leak into the bloodstream. Alcohol and its metabolites have various effects on tissues and organs, including cardiovascular effects[2] such as increased blood pressure[3], induced arrhythmias[4-6], and sudden death[7,8]. The acute intake of alcoholic beverages also causes severe cardiac damage, including cardiac arrhythmias[9,10], tissue injury related to apoptosis[11], and ultimately heart failure[12]. Alcoholic cardiomyopathy (cardiac damage caused by chronic alcohol intake) typically presents as idiopathic dilated cardiomyopathy with ventricular enlargement that ultimately impairs blood circulation[13]. It was suggested that alcohol-related sudden death often occurs on Monday (also known as holiday heart) and is associated with fatal arrhythmias, in which a tachyarrhythmia develops after alcohol consumption on the weekend[14,15]. The roles played by the sympathetic nervous system and cytokine-mediated intracellular signaling pathway have attracted attention as potential mechanisms involved in sudden death[16].

We showed that after repetitive alcohol use, changes were observed in the gene profiles related to Janus kinases (JAKs) and the signal transducer and activator of transcription (STAT), which are constituent elements of the cytokine receptor mechanism that plays an important role in controlling myocardial cell death during cardiac failure and myocardial remodeling. We also demonstrated that changes in gene profiles related to JAK/STAT activation may prevent alcohol-induced cardiomyocyte injury. However, the presence or absence of alcohol in the blood appears to alter JAK/STAT pathway activity. Indeed, acute alcohol stimulation may affect JAK/STAT pathway activity[17].

Accordingly, the present study comprehensively analyzed the gene expression profiles of myocardial tissues from a mouse model of alcohol feeding using a microarray technique. We further clarified the effect of alcohol on the myocardium from the perspective that the acute phase of alcohol use causes changes in gene expression profiles related to myocardial injury, while the withdrawal phase is associated with changes in gene expression profiles related to myocardial protection.

Materials and Methods

Animals

Pathogen-free, 7-week-old, male C57BL/6N mice (body weight 20-25 g) were obtained from SLC Inc. (Shizuoka, Japan). A mouse model of single alcohol feeding was generated by the per-oral injection of 7.0 g/kg ethanol (single group) in the alcohol-fed group or saline in the con-

trol group. A mouse model of repeated alcohol feeding was generated following Lieber's method[18]. The alcohol-fed group was fed a liquid diet containing 4% ethanol for 6 weeks (repeated group) or a control liquid diet (control group). To investigate the effects of alcohol, hearts from the alcohol-fed group were extirpated 1 h (alcohol group) or 24 h (withdrawal group) after the last alcohol feeding. The Animal Care Committee of Nagasaki University approved the research protocol (Approval No. 130520-2).

RNA extraction and gene expression analysis by real-time RT-PCR

The left ventricles of hearts were immediately dissected after animals were decapitated. The tissues were immersed in RNAlater (Ambion, CA, USA) overnight at 4℃ and then preserved at −80℃. After the tissue was homogenized using a Tissue Lyser (Qiagen, Hilden, Germany), total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA sample was stored at −80℃ until use. Total RNA (500 ng) was reverse-transcribed using the PrimeScript™ RT Reagent Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in a 10-μl reaction mix using SYBR Premix Ex Taq II (Takara Bio) and Step One™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The contents of the amplification mix and thermal cycling conditions were set according to the manufacturer's instructions. The primers for c-Fos, FBJ murine osteosarcoma viral oncogene homolog B (FosB), (connective tissue growth factor) Ctgf, tissue inhibitor of matrix metalloproteinase (Timp)-1, matrix metalloproteinase (Mmp)-3, and actin beta (Actb) were purchased from Applied Biosystems. Real-time PCR was performed at least twice for each sample. The second derivative maximum method was used for the relative quantification of mRNA transcripts. The expression levels of the target transcripts are described as the ratios of the targets normalized to the endogenous reference (Actb). The values are presented as the means ± standard error (SE), and they were analyzed by one-way ANOVA. P values <0.05 were considered statistically significant.

Blood cytokine concentrations

Blood samples were collected via the carotid artery immediately after the animals were decapitated and then stored at −80℃. Using the Bio-Plex multiplex assay system (Bio-Rad, Hercules, CA, USA), which involves xMAP technology with fluorescent microbeads, the serum samples were assayed and analyzed for various signal transducers, including 22 cytokines. The values are presented as the means ± SE, and they were analyzed by one-way ANOVA. P values <0.05 were considered statistically significant. The assayed signal transducers were: interleukin (IL)-1 α, IL-1 β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-CSF (GM-CSF), interferon (IFN)-γ, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α, MIP-1 β, regulated on activation normal T cell expressed and secreted (RANTES), and tumor necrosis factor alpha (TNF α).

Microarray

In the repeated group mice, the extirpated myocardia were homogenized using a TissueLyser (Qiagen). Total RNA was then extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen). Using RNA extracted from the extirpated myocardium as samples, mRNA expression was comprehensively analyzed using a one-color microarray technique (n=4), with SurePrint G3 Mouse Gene Expression 8x60K (Agilent, Santa Clara, CA, USA) containing 38,640 gene probes as the microchip.

GeneOntology (GO) analysis

GeneSpring GX microarray data analysis software was used to analyze the signals measured by microarray. The first step was to probe for genes with actual variations in expression (analysis of variations in expression). The gene clusters that showed variations in expression were then subjected to GO analysis. The Biobase Knowledge Library (BKL) manual curation was used for the GO Term data.

Gene cascade analysis

Gene expression cascade analysis was conducted on fragments obtained per kilobase of exon per million mapped fragment (FPKM) values, and genes related to quercetin treatment were analyzed. The analysis involved 2 steps, transcription factor binding site analysis (TFBS analysis) and key node analysis. Following the method of Dillies et al., FPKM values were normalized against the trimmed mean of M-values (TMM) method[19] and extracted into 2 groups: genes that showed changes (Yes-set), and genes that did not show changes (No-set). TFBSs included in both sets were compared, and binding sites that were significantly included in the Yes-set were searched in the BIOBASE TRANSFAC Professional database (Biobase GmbH, Wolfenbüttel, Germany). TFBS analysis was performed to predict the binding factors and each gene. Furthermore, to search for factors that influenced changes in gene expression (key nodes), key node analysis was performed by extracting the Yes-set from the obtained related genes and searching for related factors upstream of the factors in a stepwise manner.

Results

RT-PCR was performed to examine changes in the mRNA expressions of c-Fos, FosB, Ctgf, Timp-1, and Mmp-3. The levels of c-Fos and FosB mRNA were increased in the single alcohol group (Fig. 1), while the levels of Ctgf, Timp-1, and Mmp-3 mRNA were increased in the repeated alcohol group (Fig. 2).

Blood cytokine concentrations

There were no significant differences in blood cytokine concentrations in the single group. Table 1 presents the serum concentrations of various signal transducers among the repeated groups (acute, withdrawal, and control liquid diet). Compared with the control group, the acute group showed significant increases in the Th2-type cytokines IL-4, IL-5 and IL-13, as well as

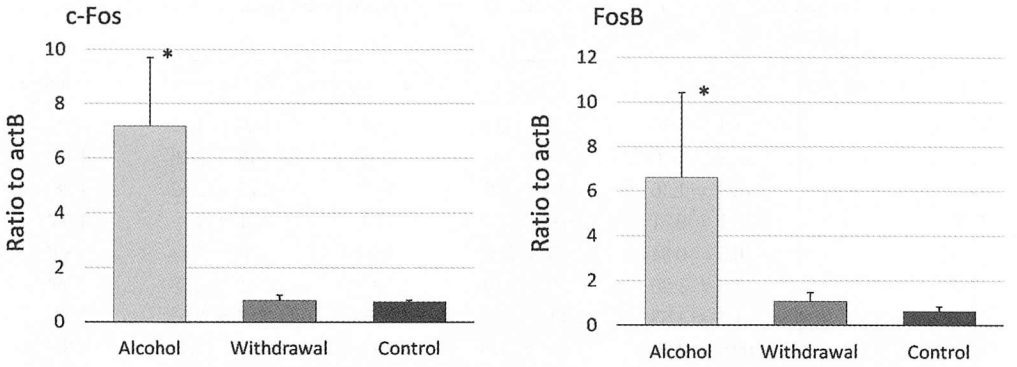

Fig 1 Heart muscle gene expression of c-Fos and FosB in the single group. Statistical significance was determined by Tukey's HSD test. *p < 0.05

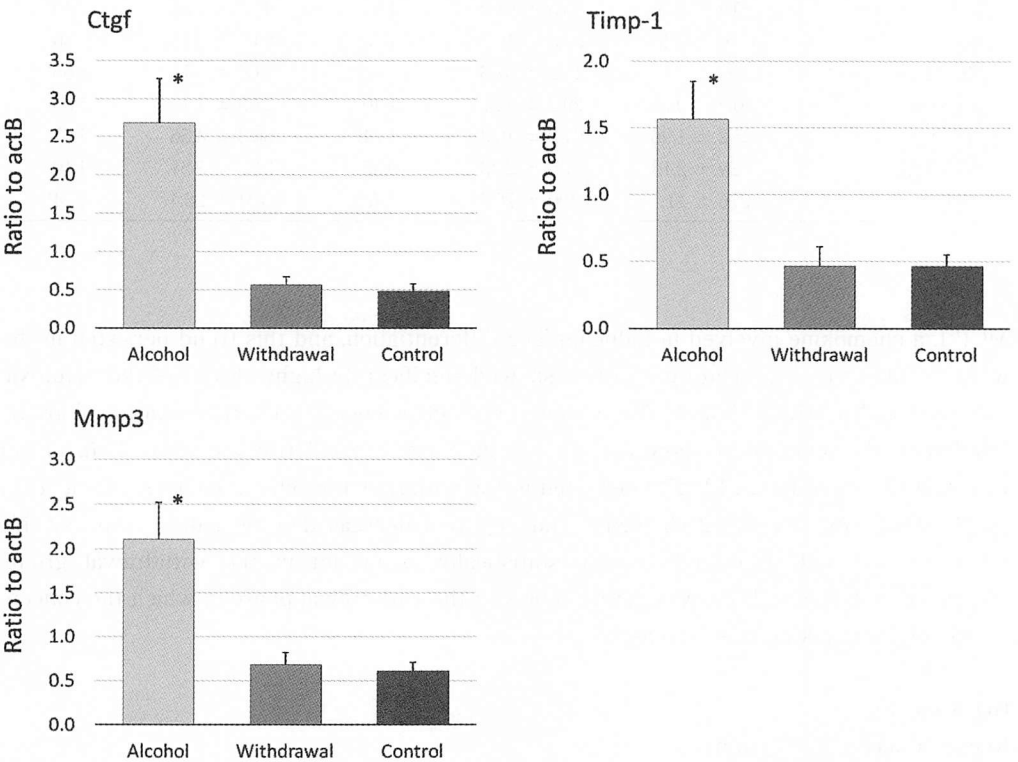

Fig 2 Heart muscle gene expression of Ctgf, TIMP-1 and MMP-3 in the repeated group. Statistical significance was determined by Tukey's HSD test. *p < 0.05

Table 1 Serum level (pg/mL) of cytokines in the repeated group. Statistical significance was determined by Tukey's HSD test. *p < 0.05

Signal Transducers	Control	Alcohol	Fold-change	Withdrawal	Fold-change
IL-1 α	8.04 ± 1.46	11.6 ± 2.81	1.44	11.9 ± 1.52	1.48
IL-1 β	308 ± 16.6	665 ± 109	2.16*	546 ± 26.6	1.77*
IL-2	18.3 ± 1.46	27 ± 1.97	1.48*	23.5 ± 2.52	1.28
IL-3	13.1 ± 0.714	19.9 ± 2.82	1.52	20.1 ± 1	1.53*
IL-4	10.5 ± 0.944	22.5 ± 3.82	2.14*	19.3 ± 1.1	1.84*
IL-5	17.6 ± 1.26	32.9 ± 5.01	1.87*	35.1 ± 2.58	1.99*
IL-6	21.4 ± 1.53	33.1 ± 6.33	1.55	30.7 ± 3.55	1.44
IL-10	69.5 ± 4.54	65.7 ± 13.4	0.95	93.5 ± 3.78	1.35*
IL-12 (p40)	364 ± 26.4	266 ± 35.1	0.73	232 ± 20.9	0.64*
IL-12 (p70)	208 ± 9.54	448 ± 83	2.15*	401 ± 27.2	1.93*
IL-13	479 ± 32.4	1230 ± 311	2.57*	812 ± 128	1.7*
IL-17	71.5 ± 9.04	79.2 ± 13.6	1.11	64.4 ± 5.14	0.90
Eotaxin	1856 ± 70.1	2092 ± 169	1.13	2297 ± 161	1.24
G-CSF	110 ± 3.69	105 ± 9.44	0.95	141 ± 14.7	1.28
GM-CSF	182 ± 8.33	206 ± 26.4	1.13	213 ± 9.37	1.17
INF- γ	29.5 ± 2.36	43.5 ± 6.92	1.47	41.2 ± 1.55	1.40
KC	62.6 ± 5.48	84.3 ± 10.3	1.35	94.1 ± 8.16	1.50
MCP-1	158 ± 13.1	272 ± 37.3	1.72*	262 ± 16.8	1.65*
MIP-1 α	20.9 ± 0.788	29.2 ± 4.27	1.40	27.9 ± 1.11	1.33
MIP-1 β	35.2 ± 1.96	61.3 ± 10.3	1.74*	56.3 ± 4.56	1.6*
RANTES	29 ± 2.13	27.5 ± 2.43	0.95	31.9 ± 3.51	1.10
TNF α	276 ± 11	490 ± 76.3	1.78*	424 ± 26.4	1.54*

MCP-1, a chemokine involved in inducing Th2 differentiation, and this trend persisted in the withdrawal group. The acute group also showed significantly higher levels of the cytotoxic cytokines IL-1 β, MIP-1 β, and TNF α, and this trend also persisted in the withdrawal group. In addition, the acute group showed significant increases in the Th1 cytokines IL-12 (p70) and IL-2, and the trend for IL-12 (p70) persisted in the withdrawal group. In contrast, the level of IL-12 (p40), a subunit of IL-12, showed a tendency to be decreased in the acute group, and this reduction was enhanced and became statistically significant in the withdrawal group. Moreover, a change in IL-10 was detected only in the withdrawal phase in which the concentration of the cytokine was increased.

GO Analysis

Repeated alcohol-fed group

Analysis of the signals in the chronic alcohol-fed group and control group identified 142 genes with significantly different gene expressions (fold change (absolute value) > 2.3 and p-value < 0.05). When gene clusters with variations in expression identified by signal analysis were subjected to GO analysis, these genes were significantly concentrated in 16 categories

Table 2 Top 3 enriched GO terms in the repeated alcohol group.

GO Term	Gene symbol	Fold enrichment	p-value
Molecular Function			
Chemokine activity	Chi3l3, Lgals3, Lmanll, Ppp1r3g, Stab2	3.7	<0.001
Endopeptidase inhibitor activity	Itih3, Papln, Serpina3n, Timp1	3.7	<0.001
Endopeptidase regulator activity	Itih3, Papln, Serpina3n, Timp1	4.3	<0.001
Biological Process			
Response to wounding	Adam8, Map2k6, Apod, Nrg1, Ctgf, Masp1, Mmp3, Il6, Itgb6, Lcn2, Lgals3, Lox, Serpina3n, Ccl17, Rtn4r, Tnfrsf12a, Timp1, Cxcl5, Ppbp, Foxo3, Ankrd1, Chi3l3, Cd59b	2.7	<0.001
Inflammatory response	Adam8, Map2k6, Masp1, Mmp3, Il6, Itgb6, Lcn2, Lgals3, Serpina3n, Ccl17, Tnfrsf12a, Timp1, Cxcl5, Ppbp, Foxo3, Chi3l3, Cd59b	2.7	<0.001
Response to external stimulus	Adam8, Dusp4, Map2k6, Apod, Nrg1, Masp1, Mmp3, Acot1, Il6, Krt8, Lgals3, Lox, Ccl17, Rtn4r, Ret, Tnfrsf12a, Timp1, Cxcl5, Ppbp, Foxo3, Cyp4f15, Chi3l3, Scd4, D0H4S114, Cd59b	2.2	<0.001
Cellular Component			
Extracellular region	Apln, Apod, Ccl17, Cd46, Chi3l3, Crlf1, Ctgf, Cxcl5, Dmkn, Fam19a3, Il6, Itih3, Lcn2, Lgals3, Lox, Masp1, Mmp3, Nppb, Nrg1, Papln, Ppbp, Serpina3n, Spaca3, Timp1	2.9	<0.001
Extracellular region part	Apln, Apod, Chi3l3, Crlf1, Ctgf, Cxcl5, Dmkn, Fam19a3, Il6, Itih3, Lgals3, Lox, Mmp3, Nppb, Nrg1, Papln, Ppbp, Serpina3n, Timp1	3.0	<0.001
Extracellular space	Apln, Apod, Chi3l3, Crlf1, Ctgf, Cxcl5, Dmkn, Fam19a3, Il6, Itih3, Lgals3, Nppb, Nrg1, Ppbp, Serpina3n, Timp1	3.0	<0.001

in the molecular function domain, 75 categories in the biological process domain, and 6 categories in the cellular component domain. Table 2 shows the top three of three categories with low p values.

Analysis of the signals in the alcohol withdrawal group and control group identified 82 genes with significantly different gene expressions (fold change (absolute value) > 2.0 and p-value < 0.05). When gene clusters with variations in expression identified by signal analysis were subjected to GO analysis, these genes were significantly concentrated in 6 categories in the molecular function domain, 16 categories in the biological process domain, and 3 categories in the cellular component domain. Table 3 shows the top three of three categories with low p values.

Table 3 Top 3 enriched GO terms in the repeated withdrawal group.

GO Term	Gene symbol	Fold enrichment	p-value
Molecular Function			
Endopeptidase inhibitor activity	Rarres1, Serpina10, Spink6	2.3	<0.01
Endopeptidase regulator activity	Rarres1, Serpina10, Spink6	2.3	<0.01
Peptidase inhibitor activity	Rarres1, Serpina10, Spink6	2.3	<0.01
Biological Process			
Myelination	Acsbg1, Adam22, Apod, Klk6	3.0	<0.01
Axon ensheathment	Acsbg1, Adam22, Apod, Klk6	3.0	<0.01
Regulation of cell proliferation	Bcat1, Car1, Crmp1, Dbc1, Dct, E2f8, Egr3, Fabp7, Gkn1, Hmga2, Klk6, N6amt1, Ppbp, Retnla, Skap1	2.4	<0.01
Cellular Component			
Extracellular space	Apod, Chi3l3, Igj, Ppbp, Retnla, Serpina10	2.6	<0.01
Extracellular region	Apod, Chi3l3, Igj, Lcn2, Ppbp, Retnla, Serpina10	2.2	0.025
Extracellular region part	Apod, Chi3l3, Igj, Ppbp, Retnla, Serpina10	1.9	0.019

Gene cascade analysis

Repeated alcohol-fed group

When gene clusters with variations in expression identified by signal analysis (142 genes) were analyzed for those that had a significant number of transcription factor binding regions located upstream of the transcription start sites of the gene clusters, 12 such regions were identified. When binding regions (12 regions) obtained by probing for transcription factor binding regions were analyzed for binding transcription factors, 12 factors were identified. A search was performed upstream from these 12 factors, and key node analysis produced 85 key node proteins. Furthermore, cascades associated with these key node proteins were examined, and proteins that appeared in all cascade diagrams at high frequencies were analyzed. Table 4 shows the top 20 factors with a high appearance rate.

Repeated withdrawal group

When gene clusters with variations in expression identified by signal analysis (82 genes) were analyzed for those that had a significant number of transcription factor binding regions located upstream of the transcription start sites of the gene clusters, 44 such regions were identified. When binding regions (44 regions) obtained by probing for transcription factor binding regions were analyzed for binding transcription factors, 42 factors were identified. A search was performed upstream from these 42 factors, and key node analysis produced 28 key node proteins. Furthermore, cascades associated with these key node proteins were examined, and

Table 4 Top 20 factors of the cascade analyses in the repeated alcohol group.

Node Name	Pathway name
Pparg	beta-catenin:E-cadherin complex phosphorylation and dissociation
ER-beta	beta-catenin:E-cadherin complex phosphorylation and dephosphorylation
Jak1	beta-catenin network
Jak2	PDGF pathway
SHP2-isoform2	IL-10 --> STAT1alpha, STAT3
INSR	IFNalpha, IFNbeta --> STAT5
Src	IL-10 pathway
gp130	IFNalpha, IFNbeta, IFNgamma --> Rap1
PDGFRalpha	PDGF B --> cortactin
SHP1-isoform1	PDGF B --> cortactin
VEGFR-2	IFNalpha, IFNbeta --> STAT1alpha, STAT2, STAT3
PDGFRbeta	OSM --> STAT1
Pdx1	OSM --> STAT3
IFNAR2	IFNalpha, IFNbeta --> PI3K
c-Kit	PDGF B --> Src
IFNAR1	PDGF A --> Src
IL-7Ralpha	PDGF B --> Src
LynA	LMW-PTP --/ VEGFR-1
LynB	PDGF A --> SHP-2
IFNAR2c	CD148 --/ PDGFRbeta

Table 5 Top 20 factors of the cascade analyses in the repeated withdrawal group.

Node Name	Pathway Name
Junb	p38 pathway
huntingtin	Stress-associated pathways
p300	Caspase network
NFKB1	Fas pathway
PKCzeta	EDAR pathway
IKK-beta	TLR3 pathway
CBP	TLR4 pathway
PKCdelta	RANKL pathway
Fosb	MEKK2, MEKK3 --> p38, JNK1
SRF-L	MLK2, MEKK1 --> c-Jun
Irf1	EDA-A2 --> c-Jun
p/CAF	MKK4, MKK7 --JNK1--> ATF-2
CaMKIV	Caspase-3 --/ p53
IKK-alpha	AKT-1 --/ JNK1
Caspase-3	BMP2 --> p38alpha
Bcl-2	MKK4 --> c-Jun
calcineurin	ASK1 --MKK7,JNK1--> c-Jun
Cebpd	MKK4, MKK7 --JNK2--> ATF-2
Ku80	JNK1 --> MKK4
Nanog	Rac1 pathway

proteins that appeared in all cascade diagrams at high frequencies were analyzed. Table 5 shows the top 20 factors with a high appearance rate.

Discussion

Stimuli to cells are transmitted via cytokine signaling through specific receptors to the intracellular compartment and then delivered into the nucleus through the signal transduction pathway. Many immediate early genes (IEGs) in the nucleus are transcription factors involved in physiological processes such as cell proliferation and apoptosis by either transcribing themselves or regulating the transcription of other genes located downstream[20,21]. The present study found high expression rates of c-Fos and FosB, IEGs activated by the MAPK pathway, in the single alcohol group. This suggests that a single instance of ethanol exposure triggered the MAPK pathway leading to an immune response while ethanol is still present in the blood. Therefore, a single instance of myocardial tissue exposure to ethanol might activate an intracellular signaling cascade mediated by the MAPK pathway. It was reported that activation of ERK1/2 in the MAPK pathway promoted cardiac hypertrophy in mice[22,23]. This finding indicates that the MAPK pathway is involved in adaptation to acute exposure to ethanol. In addition, alcohol use disorders and heavy drinking have been associated with the mainly pro-inflammatory effects of alcohol[24]. Chronic alcoholism, complicated by alcoholic liver disease, was shown to be characterized by alterations in the balance between pro-inflammatory and anti-inflammatory cytokine signaling[25]. Studies in animal models have shown that some immune proteins act as both immune-signaling molecules in the periphery and signaling molecules in the brain[26].

In general, the heart dilates to adapt to mechanical stressors, such as pressure and volume loading, and other types of stress. However, this adaptation fails when the stress becomes excessive or prolonged, leading to heart failure from cardiomegaly, cardiac dysfunction, myocardial cell death, or fibrosis[27]. The mechanism of cardiac hypertrophy and heart failure involves the extracellular matrix, a tissue structure that connects cardiomyocytes and relays mechanical tension, and as such plays an important role in dilating the ventricular chamber[28]. MMPs, a group of enzymes that degrade the extracellular matrix, are regulated by endogenous inhibitory proteins known as TIMPs[29]. Patients with heart failure show increased expressions of proteins that activate extracellular MMP, indicating that an imbalance between the synthesis and degradation of the extracellular matrix is one of the causes involved in cardiomegaly observed in heart failure. In the present study, the expressions of Ctgf and Timp-1, factors regulating MMPs, were higher in the repeated alcohol group. Although increased levels of these factors should have an inhibitory effect on MMPs, an increase in Mmp3 expression was detected in the repeated alcohol group. This suggests that during repeated ethanol exposure, MMP3 becomes active and disengaged from the inhibitory control of Ctgf and Timp-1 when ethanol is still present in the blood. The heart compensates for stimulation through hypertrophy aided by the suppression of MMP3 while it is still able to adapt, but heart failure occurs when the inhibitory control of MMP3 collapses[30]. Therefore, it can be posited that repeated

ethanol stimulation triggers a myocardial compensation mechanism modulated by Ctgf, Timp-1, and Mmp3 in mouse myocardial tissue.

In the present study, no changes in blood cytokine levels were seen in the single alcohol group. This indicates that a single instance of alcohol consumption has no systemic effect on signal transducers. Alcohol consumption increased the risk of developing type I allergies through increased total IgE and other effects[31]. The onset and aggravation of type I allergies are highly associated with immune responses mediated by Th2 cells, and the present study detected an increase in Th2-type cytokines and chemokines in the blood of the repeated alcohol group. Therefore, the repeated feeding of alcohol might generate a Th2-driven immune reaction. However, alcohol can trigger the release of cytotoxic cytokines by acting directly on Kupffer cells and macrophages, and levels of blood cytotoxic cytokines were higher in the repeated alcohol group. This implies that repeated exposure to alcohol resulted in acute alcohol stimulation that activated monocytes, macrophages, and other related types of cells. Although activation of monocytes and macrophages leads to an increase in IL-12 levels, a cytokine responsible for inducing Th1 differentiation, chronic alcohol consumption shifts the immune response toward Th2 by suppressing IL-12. In the repeated alcohol group, levels of IL-12 (p70) and IL-2 were significantly higher, but the level of IL-12 (p40), a subunit of IL-12, was lower. This suggests that repeated alcohol exposure might suppress IL-12 production. Moreover, alcohol promotes IL-10 production and thus reduces IL-12 levels, because IL-10 is an inhibitory cytokine that restricts the synthesis of other pro-inflammatory cytokines. In the repeated alcohol groups, an increase in IL-10 was seen only in the withdrawal group. Therefore, the immune response might shift toward Th2 as IL-10 production increased when blood alcohol reached a level of elimination following repeated alcohol feeding.

Although cases of sudden death in heavy drinkers are common in forensic practice, the findings often consist only of low blood ethanol levels and mild hepatic steatosis, and significant morphological changes that would explain the cause of death are rarely discovered. Thus, it has been suggested that fatal arrhythmias are linked to chronic excessive alcohol use, with intracellular signaling pathways involved in the mechanism. The present study investigated the effects of chronic alcohol use on myocardial tissues by comprehensive gene expression profiling and analyses of myocardial tissues from the acute and withdrawal phases of a mouse model of chronic alcohol use. Significant changes in the gene expression profiles were seen in the repeated alcohol group and the repeated withdrawal group compared with the repeated control group, which demonstrated that gene expressions in myocardial tissues varied by chronic alcohol use in the acute and withdrawal phases.

Significantly large variations in gene expressions were seen in 142 genes in the chronic alcohol-fed group and 82 genes in the alcohol withdrawal group (gene clusters with variations in expressions). This might be related to the result of chronic alcohol use. GO analysis of the gene clusters with variations in expression showed that in the acute phase, genes in which there were variations in expression were primarily associated with cytokines/chemokines and with the inflammatory response, while in the withdrawal phase, they were primarily associated with proteases. These findings suggest that chronic alcohol use produces inflammatory

responses mediated by cytokines and chemokines in the acute phase and protease-mediated cell proliferation in the withdrawal phase.

However, when the gene clusters with variations in expressions were probed for those with significantly more transcription factor binding regions located upstream of the transcription start sites of these genes compared with those with no variations in expression, there were 12 transcription factor binding regions in the chronic alcohol-fed group and 44 in the alcohol withdrawal group. Furthermore, key node analysis of the transcription factors binding in these regions identified 85 key node proteins in the repeated alcohol group and 28 in the repeated withdrawal group. Gene cascade analysis of these key node proteins showed a tendency for activation to hypercardia with the STAT family, and to apoptosis with p38 and the caspase pathway in the alcohol withdrawal group. In myocardial tissue, chronic alcohol use may promote transcription of mRNAs related to pathways associated with hypercardia in the acute phase, and that the activity of pathways associated with the STAT family may not be maintained in the withdrawal phase, with the transcription of mRNAs of p38 and caspase pathways mediated by apoptosis in the withdrawal phase.

Conclusion

The present study used a microarray technique to perform a comprehensive analysis of gene expression profiles of myocardial tissues from a mouse model of alcohol use. Differing gene profiles were seen in the repeated groups, which suggested that activation of the STAT pathway and the cytokine-mediated inflammatory response to hypercardia may occur in the acute phase, while in the withdrawal phase STAT activity may not persist although apoptosis may occur and be related to the development of sudden death.

In the future, after conducting more detailed analyses of the gene profiles, we plan to investigate the effects of alcohol on cardiomyocytes in greater detail by hypothesizing which proteins are the source of the variations in gene expressions and conducting individual analyses of gene expression and proteins.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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