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Original

Expression of RAE-1, a ligand of the NKG2D Receptor, in Mice Adipocytes

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Abstract : Although epidemiological evidence has confirmed the role of heavy alcohol consumption as an established risk factor for esophageal, liver, and breast cancers, the molecular mechanisms underlying this effect are not fully understood, particularly with regard to breast cancer. In this study, expression of the *retinoic acid early inducible-1* (*RAE-1*) gene was determined in mice adipocytes. RAE-1 is a ligand of the natural-killer group 2 member D (NKG2D) receptor, which plays a crucial role in tumor immunity. RT-PCR and Western blotting analysis demonstrated that TNF-α treatment induced *RAE-1* mRNA and RAE-1 protein expression in adipocytes obtained from differentiated 3T3-F442A cells. Real-time RT-PCR analysis showed that 300 mM ethanol enhanced *RAE-1* mRNA expression, which peaked 6 h after administration. In addition, *RAE-1* mRNA was detected in visceral adipose tissue obtained from mice. These observations indicate that *RAE-1* mRNA is expressed in adipocytes and that its expression is enhanced by TNF-α, which has been shown to be induced in adipose tissue by long-term alcohol consumption. Furthermore, ethanol directly enhanced *RAE-1* mRNA expression, suggesting that alcohol consumption enhances RAE-1 expression in adipose tissue and might modify tumor immunity. This finding might provide novel insight into the mechanism of alcohol-associated carcinogenesis.

Key words : RAE-1, NKG2D receptor ligand, adipocyte, tumor immunity, ethanol

Introduction

Epidemiological evidence has confirmed the role of alcohol consumption as an established risk factor for esophageal, liver, and breast cancers[1]. Many studies have investigated the molecular mechanisms of carcinogenesis associated with alcohol consumption. Alcohol dehydrogenase 2 (ALDH2) genotypes are closely associated with the incidence of esophageal cancer. Acetaldehyde, a metabolite of ethanol formed via ALDH2 activity, is a potent carcinogen associated with esophageal cancer[2]. In addition to acetaldehyde, some reactive oxygen species (ROS) are also formed, and these intermediate metabolites of ethanol

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attack DNA, resulting in carcinogenesis in hepatocellular carcinoma[2]. Furthermore, alcohol consumption enhances the absorption of endotoxins from the intestinal flora, which evoke inflammation in Kupffer cells and subsequently promote hepatocellular carcinoma[3].

Although alcohol consumption also increases the risk for breast cancer, the mechanisms underlying this effect remain to be clarified. Some studies have reported that long-term estrogen exposure contributes to carcinogenesis, and that alcohol consumption is possibly associated with estrogen secretion[4]. Ben-Eliyahu *et al* reported that alcohol intake decreased natural killer (NK) cell activity, which resulted in a consequent 10-fold increase in the metastasis of breast cancer cells to the lung in an *in vivo* experimental model[5]. These results suggest that the alteration of tumor immunity by alcohol consumption could be associated with the development of breast cancer.

Natural-killer group 2 member D (NKG2D) receptors are expressed on the membrane of all NK cells, most NKT cells, and subsets of $\gamma\delta$ T cells[6]. NK cells mount a major anti-tumor response by recognizing and eliminating tumor cells through connecting NKG2D receptors with their ligands on target cells[7]. Cancer cell proliferation is markedly enhanced in NKG2D receptor-deficient mice compared to wild-type mice, indicating the critical role in cancer immunity played by the response of NKG2D receptor-ligands[8].

Many ligands have been described for the NKG2D receptor in mice. One ligand is retinoic acid early inducible-1 (RAE-1)[9]. Under physiological conditions, RAE-1 expression in tumor cells exerts a suppressive effect on tumor proliferation[10]. However, transgenic mice overexpressing RAE-1 specifically in the skin were more susceptible to chemically induced tumors, possibly due to the suppression of tumor immunity[11]. In addition, transgenic mice with overexpression of MICA (MHC class I chain-related protein A), a human NKG2D ligand, showed that NKG2D receptor levels were decreased in $CD8^+$ T cells[12]. These results indicate that the maintenance of tumor immunity requires that NKG2D ligands be strictly expressed only in tumor cells targeted by NK cells, and that when they are ectopically expressed, tumor immunity might be suppressed.

RAE-1 is expressed in the early stages of embryonic development[9]. In adult mice, RAE-1 is constitutively expressed in the liver and spleen, suggesting that these organs regulate NK cell activity and tumor immunity[10]. Many recent studies have reported that adipocytes have multiple physiological and pathophysiological functions[13]. This study, therefore, investigated the expression of RAE-1 in adipocytes, and examined whether ethanol modified the expression levels of *RAE-1* mRNA.

Materials and Methods

Cell Culture

3T3-F442A cells were purchased from European Collection of Cell Cultures (ECACC, UK). These cells were cultured at 37°C and 5% CO_2 in DMEM (Gibco, USA) containing 10% fetal bovine serum (Biowest, France)[14]. These cultured preadipocytes were maintained

for 7 days at 100% confluency, and were stimulated to differentiate into adipocytes by treatment with 5 μg / ml Recombinant Human Insulin (Gibco, USA). Seven days after treatment with insulin, the presence of mature adipocytes was confirmed by Oil Red O staining.

RT-PCR and Quantitative Real time RT-PCR

Epididymal adipose tissues and spleens were obtained from male BALB / cAJcl mice (CLEA Japan Inc., Japan). Adipose tissues and 3T3-F442A adipocytes were washed with PBS, and total RNA was extracted using TRIzol Reagent (Invitrogen), then purified using the RNeasy Mini Kit (Qiagen, USA). cDNA was prepared using the ReverTra Ace qPCR RT kit (Toyobo, Japan). Real-time PCR was performed using SYBR® Green I and Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA). The PCR cycling conditions involved 45 cycles consisting of 95℃ for 15 s and 60℃ for 1 min. Primers used for analysis of *RAE-1* mRNA expression were : sense, 5'-CCACCTGGGAATTCAACATC-3' ; and antisense, 5'-CCCCTGATTCATCATTAGCTG-3'. The integrity of the cDNA was assessed by amplification of 18S rRNA using the following primers : sense, 5'-AAACGGCTACCACATCCAAG-3' ; and antisense, 5'-CAATTACAGGGCCTCGAAAG-3'.

Western blotting

3T3-F442A adipocytes were homogenized in RIPA buffer (25 mM Tris / HCl pH7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS ; Thermo Fisher Scientific Inc., USA) containing Complete Mini protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA). The proteins (20 μg) were separated by 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Hybond-ECL ; GE Healthcare UK Ltd., Buckinghamshire, UK). The membranes were blocked at room temperature for 1 hour in 5% non-fat milk powder in TBS-T (10 mM Tris base pH7.5, 450 mM NaCl, and 0.1% Tween 20) and then incubated overnight at 4℃ with the anti-mouse RAE-1 polyclonal antibody (sc-20333 ; Santa Cruz Biotechnology, Inc., USA). After incubation with the secondary antibody donkey-anti-goat IgG-HRP (sc-2020 ; Santa Cruz Biotechnology, Inc., USA), immunoreactive bands were visualized using enhanced chemiluminescence reagents (ECL Plus Western Blotting Detection Reagent ; GE Healthcare UK Ltd.).

Results

Effect of TNF-α treatment on the expression of RAE-1 mRNA and RAE-1 protein in 3T3-F442A adipocytes

After confirming the differentiation of 3T3-F442A cells into mature adipocytes by Oil Red O staining (Fig. 1A), the ability of TNF-α to induce *RAE-1* mRNA and RAE-1 protein

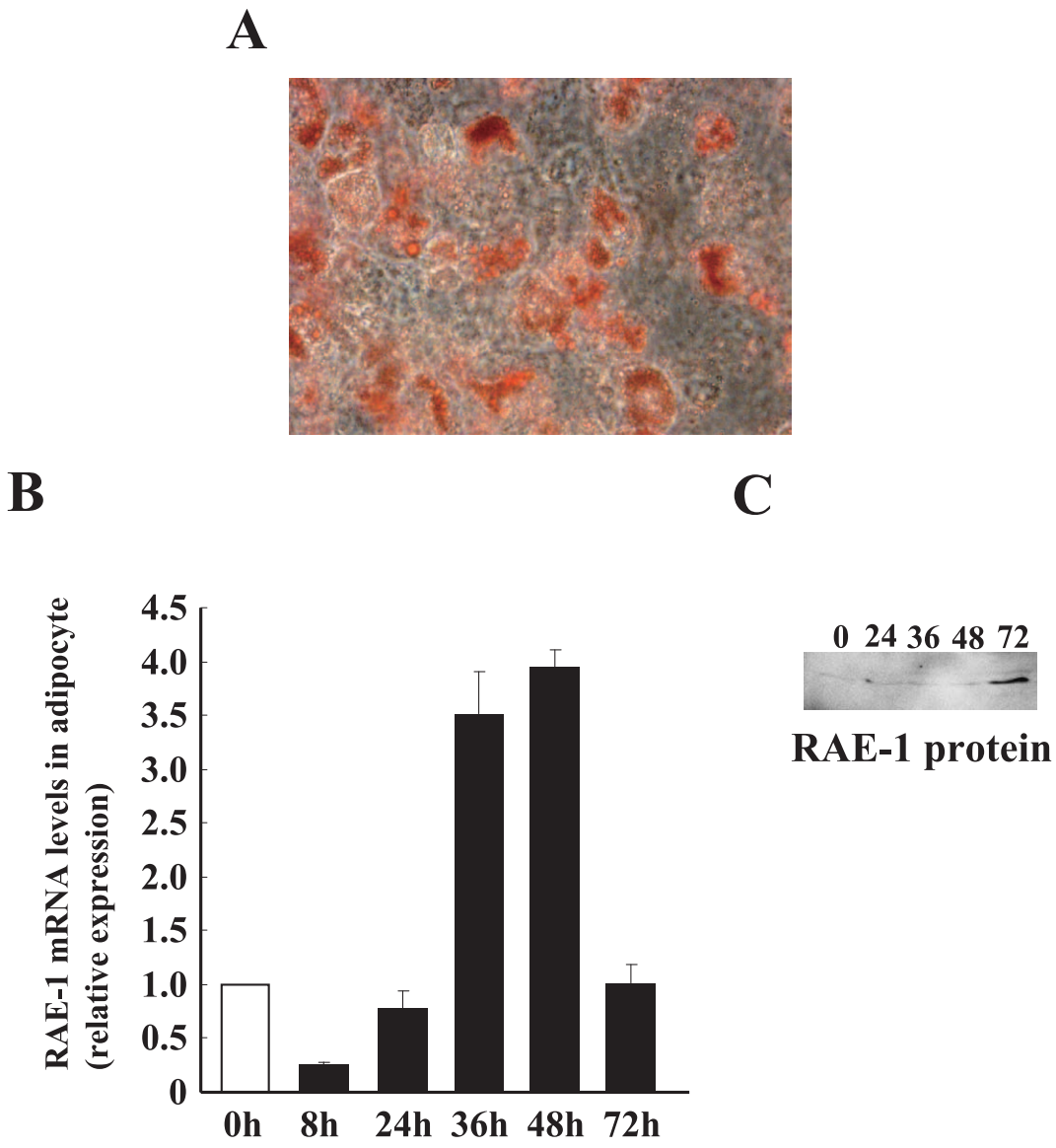

Fig. 1. Time-course of changes in *RAE-1* mRNA and RAE-1 protein levels after treatment of 3T3-F442A-derived adipocytes with TNF-α

A) The presence of mature adipocytes, obtained by differentiating 3T3-F442A cells in the presence of insulin, was confirmed by Oil Red O staining. Fat was stained red. B) Time-course of changes in *RAE-1* mRNA expression levels after treatment with TNF-α (50 ng / ml). Real-time PCR revealed that the level of *RAE-1* mRNA increased 4 fold to peak levels at 48 h after treatment, and decreased to pretreatment levels by 72 h. C) Time-course of changes in RAE-1 protein levels following treatment with TNF-α. Western blotting revealed that RAE-1 protein levels markedly increased to peak levels at 72 h.

expression in these adipocytes was tested. Treatment with 50 ng / ml TNF-α induced the expression of *RAE-1* mRNA. Fig. 1B shows the time-course of changes in *RAE-1* mRNA expression levels after treatment with 50 ng / ml TNF-α. *RAE-1* mRNA expression initially

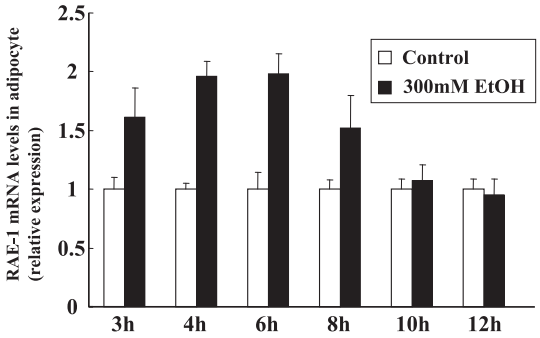

Fig. 2. Time-course of changes in *RAE-1* mRNA levels after treatment with ethanol
Time-course of changes in *RAE-1* mRNA expression levels after treatment with ethanol (300 mM) was determined by real-time RT-PCR.

decreased 8 h following treatment, then increased almost 4 fold to peak levels 48 h after treatment, before returning to the pretreatment level. Western blotting demonstrated that the RAE-1 protein level did not change until 48 h after treatment with TNF-α, and was markedly increased at 72 h (Fig. 1C)

Effect of ethanol treatment on RAE-1 mRNA levels in 3T3-F442A adipocytes

Quantitative time-course changes in the mRNA expression of *RAE-1* were examined after treating adipocytes differentiated from 3T3-F442A cells with 300 mM ethanol. Preliminary experiments showed that treatment with 100 mM ethanol did not change the expression levels of *RAE-1* mRNA (data not shown). Fig. 2 shows that the expression levels of *RAE-1* mRNA increased from 3 h after treatment, peaked at 6 h, and then returned to the control levels 10 h after treatment.

Expression of RAE-1 mRNA in the adipose tissue of BALB/cAJcl mice

The expression of *RAE-1* mRNA in the adipose tissue of mice was examined *in vivo*, and the results were compared to the physiological levels found in the spleen[15]. Although *RAE-1* mRNA levels were lower in adipose tissue than in the spleen, *RAE-1* mRNA was constitutively expressed in adipose tissue (Fig. 3).

Discussion

To our knowledge, this is the first report demonstrating that the *RAE-1* gene, a ligand of the NKG2D receptor, is expressed in adipocytes. Our data showed that *RAE-1* mRNA was constitutively expressed in adipose tissue *in vivo*, and that expression of *RAE-1* mRNA in 3T3-F442A-derived adipocytes was up-regulated by TNF-α and ethanol. These findings suggest the possibility that adipose tissue might be involved in tumor immunity, and that

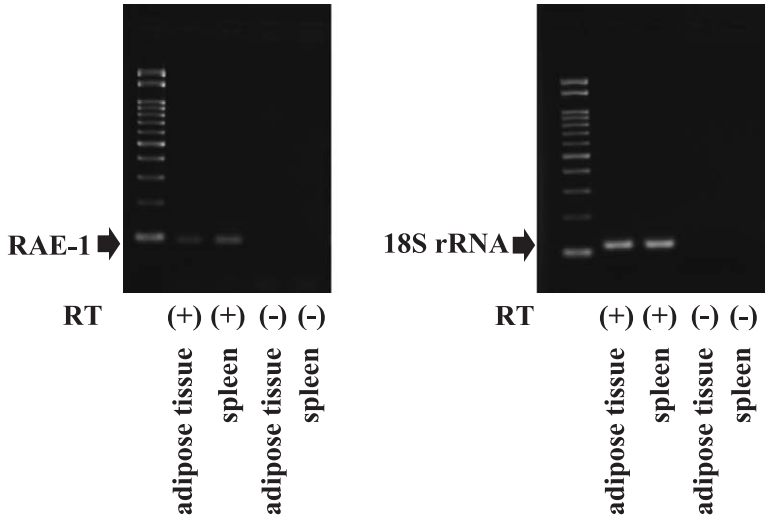

Fig. 3. Comparison of *RAE-1* mRNA expression in adipose tissue and mice spleens *in vivo*

Constitutive expression of *RAE-1* mRNA in adipose tissue was determined by RT-PCR, and compared to expression levels in the spleen of mice. RT (+) indicates that the reverse transcription reaction was performed on the template. RT(−) indicates that the reverse transcription reaction was not performed on the template. This was included as the negative control.

alcohol consumption might modify NK cell activity through the alteration of RAE-1 expression in adipocytes.

Chronic alcohol consumption induces inflammation and TNF-α secretion in adipose tissue[16, 17]. Fig. 4 shows a hypothetical schematic illustration of ethanol-induced expression of RAE-1 in adipose tissues. TNF-α might induce the release of saturated fatty acids from adipocytes, which then activate macrophages through toll-like receptor 4 (TLR4), resulting in the induction of TNF-α. This cycle leads to sustained chronic inflammation of the adipose tissue[17]. Our results revealed that TNF-α enhances *RAE-1* mRNA and RAE-1 protein expression, suggesting that RAE-1 expression might be involved in sustained chronic adipose tissue inflammation by activating NK cells in the adipose tissue.

In vivo experiments have demonstrated that the administration of ethanol disturbs the activity of NK cells[18]. In this study, treatment with ethanol caused a 2-fold increase in the level of *RAE-1* mRNA in adipocytes. This elevation was not seen until 6 h after treatment. However, increases in *RAE-1* mRNA levels after treatment with TNF-α occurred later than with ethanol, suggesting that the *RAE-1* gene signal transduction pathway might differ between ethanol and TNF-α. A previous study demonstrated that an oral administration of ethanol decreased NK cell activity, peaked at 12 h[18], and increased the metastasis of cancer cells within 24 h[5]. These findings suggest that the administration of ethanol might directly suppress tumor immunity, and the time-course of this phenomenon is consistent with the

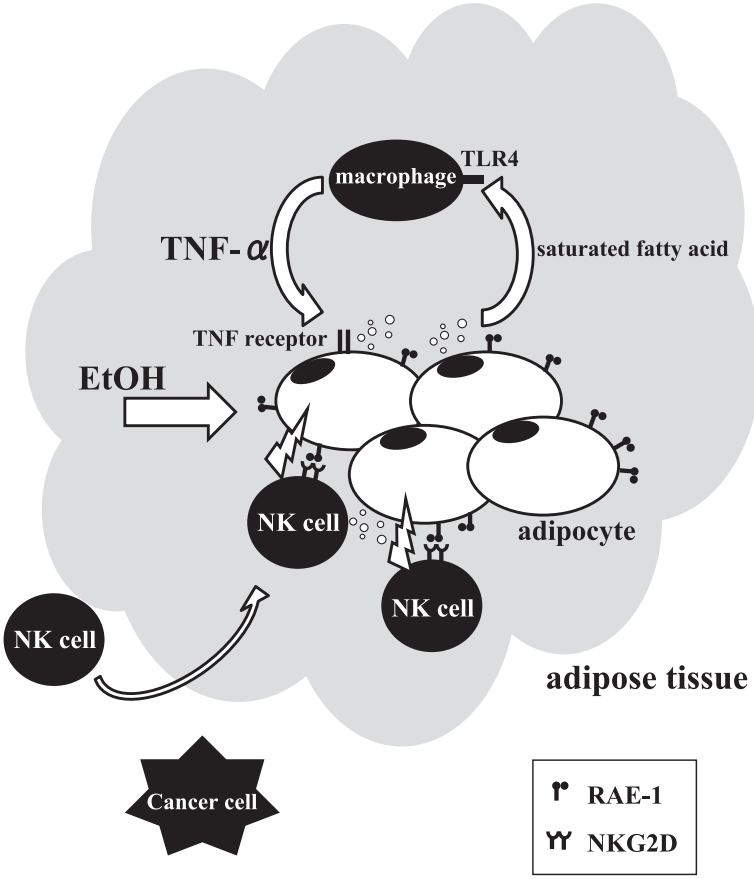

Fig. 4. Schematic illustration demonstrating the hypothesis that ethanol-induced expression of RAE-1 in adipose tissues results in disturbed tumor immunity

Chronic alcohol consumption induces inflammation and TNF-α secretion in adipose tissue. TNF-α is a key molecule in the induction of RAE-1. TNF-α induces the release of saturated fatty acids from adipocytes, which then activate macrophages through toll-like receptor 4 (TLR4), resulting in the induction of TNF-α. This cycle leads to sustained chronic inflammation in adipose tissue and elevation of TNF-α. TNF-α, or ethanol (or both), and enhances the expression of RAE-1, which draws circulating NK cells into the adipose tissue. These NK cells are activated and cause inflammation in the adipose tissue via the binding of NAG2D receptors on the NK cell with the RAE-1 ligand on the adipocytes. The number of NK cells available to attach to tumor cells is consequently decreased and tumor immunity is disturbed.

elevation of *RAE-1* mRNA expression following ethanol treatment.

These results suggest that chronic alcohol consumption might elevate RAE-1expression either directly or indirectly (by TNF-α induction), or both. It is hypothesized that RAE-1 expression in adipocytes can draw circulating NK cells into the adipose tissue. Subsequently, the interaction of NKG2D receptors on the NK cell with the RAE-1 ligand on the adi-

pocytes activates the NK cells and causes inflammation in the adipose tissue. The number of NK cells available for attachment to tumor cells is consequently decreased and tumor immunity is disturbed (Fig. 4).

It is well known that TNF-α is induced in adipocytes and in macrophages infiltrating into adipose tissue in obese mice[17]. It has thus been suggested that obesity and alcohol consumption are independent risk factors for breast cancer. However, the current findings may suggest that obesity and alcohol consumption share a similar molecular mechanism in the carcinogenesis of breast cancer.

In this study, the expression of RAE-1, a ligand of the NKG2D receptor, was identified in adipocytes. Many recent studies have reported that adipocytes have multiple functions, including the secretion of several physiological activating factors such as adiponectin, interleukins, and growth factors[13]. The data from this study suggest the possibility that adipose tissue might play an important role in the regulation of tumor immunity. Alcohol consumption might modify this immunity by the induction of RAE-1, a ligand for the NKG2D receptor. This hypothesis may provide novel insight into the mechanisms underlying alcohol-associated carcinogenesis.

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