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γ -Secretase inhibitor reduces diet-induced atherosclerosis in apolipoprotein E-deficient mice

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Abstract

Atherosclerosis is a chronic inflammatory disease resulting from interactions between lipids, macrophages and arterial wall cells. The Notch signaling pathway is involved in the activation of macrophages in atherosclerotic lesions. This study examined whether pharmacological inhibition of Notch signaling using a γ -Secretase inhibitor (GSI) can reduce atherosclerotic lesion formation. Notch-related molecules were significantly increased in aortas from apolipoprotein E-deficient (ApoE^{-/-}) mice. In particular, macrophages in the plaques showed strong expression of Notch1 and a downstream transcriptional factor, Hes-1. A GSI (LY411,575, 0.2, and 1.0 mg/kg/day) or vehicle control was then administered to ApoE^{-/-} mice fed Western diet for 8 weeks before measuring the expression of Notch-related molecules. Systemic administration of GSI suppressed Notch signaling *in vivo* and reduced total plaque areas and fatty streak content in the aortic sinus in a dose-dependent manner without serious adverse effects. The GSI also suppressed the migratory activity of macrophages and reduced the expression of intercellular adhesion molecule-1, resulting in significantly decreased macrophage infiltration in the atherosclerotic plaques. These results provided new insight into the anti-atherogenic properties of GSI in ApoE^{-/-} mice fed Western diet.

Keywords

Atherosclerosis; Inflammation; γ -Secretase inhibitor

Introduction

Macrophages exacerbate inflammatory responses in atheromatous plaques and promote their structural instability [1]. The inflammatory response could therefore be a critical target in atheromatous lesions to prevent atherogenesis [2,3].

Notch receptors are highly conserved, membrane-bound receptors that regulate binary cell fate during development and adulthood [4]. We reported previously that endothelial Notch1 plays a critical role in vasculogenesis during embryogenesis [5] and in angiogenesis in response to ischemia in adults [6]. Notch signaling has also been extensively implicated in cell-fate selection throughout immune development, particularly in the development and differentiation

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of lymphocytes and monocytes [7–9] and in regulating the functions of mature immune cells. Notch1 signaling is associated with macrophage activation via upregulation of expression of intercellular adhesion molecule 1 (ICAM-1) and major histocompatibility class II [10]. Notch signaling is therefore intricately involved in activating the immune system and would similarly affect the inflammatory process in atherosclerosis. Indeed, Aikawa et al. [11,12] showed that Notch ligands and receptors are highly expressed in macrophages from atherosclerotic lesions where they could mediate the inflammatory response. Thus, we hypothesized that Notch signal activation in immune cells exacerbates atherosclerotic lesions and in turn that inhibition of Notch signaling with a γ -Secretase inhibitor (GSI) prevents or suppresses atherogenesis.

GSIs are potential therapeutic agents for Alzheimer's disease based on their action in preventing the enzymatic cleavage of amyloid precursor proteins and consequently decreasing amyloid β production [13]. GSIs also block Notch intracellular domain production both *in vitro* [14] and *in vivo* [15], thus also suppressing Notch signaling. GSI treatment recently decreased leukocyte infiltration and neural cell damage in a murine stroke model [16]. The present study thus was designed to determine whether GSI treatment can suppress the progress of atherogenesis in apolipoprotein E-deficient (ApoE^{-/-}) mice.

Materials and methods

Animals and diets

Six-week-old male ApoE^{-/-} mice with a C57BL/6 J background (Jackson Laboratory, Bar Harbor, ME) were fed an atherogenic diet (Western diet = 21% fat and 0.15% cholesterol) for 8 weeks. LY411,575 was used as a γ -Secretase inhibitor compound based on its potent inhibition of Notch S3 cleavage and its bioavailability [17]. The mice were randomly assigned to receive either vehicle alone (0.4% methylcellulose) or LY411,575 (0.2 or 1.0 mg/kg/day) orally during the Western diet. Adverse effects were monitored by recording mortality, incidence of diarrhea, body weight, and plasma lipid levels. Plasma total cholesterol, triglyceride, and high-density lipoprotein levels were measured with a commercially available enzymatic kit (Wako, Japan). Following perfusion-fixation, samples of ileum and thymus were processed for paraffin embedding, and sections were stained with periodic acid-Schiff (PAS) and hematoxylin-eosin (H&E) to assess intestinal goblet cell hyperplasia and thymic atrophy, respectively, as indicators of minor adverse effects [15,18]. The Animal Studies Committee of Nagoya University Graduate School of Medicine approved the experimental protocol.

Quantification of Notch-related molecules

Total RNA was extracted from the thoracic aortas of wild-type and ApoE^{-/-} mice after treatment and reverse-transcription, as described previously [19]. The synthesized cDNA of each gene was quantified using a SYBER Green RT PCR kit (Qiagen, Hilden, Germany) as described previously [19]. The primer and probe sequences used in this study were as follows: mouse Notch1, (forward) 5'-tgtgacagccagtgaactc, (reverse) 5'-tggcactctggaagcactgc; mouse Notch3, (forward) 5'-acactgggagtctctgt, (reverse) 5'-gtctgctggcatgggata; mouse Hes-1, (forward) 5'-gccagtgtcaacacgacaccgg, (reverse) 5'-tcacctcgttcactcactcg; mouse Jagged-1, (forward) 5'-attcgatctacatagcctgtgag; (reverse) 5'-ctatacgatgtattccatccgggt; mouse β -actin, (forward) 5'-tggaatcctgtggcatccatgaac, (reverse) 5'-taaacgcagctcagtaacagtcgcg. RNA amounts were each normalized to their respective β -actin mRNA.

Immunohistochemical analysis

Immunohistochemistry using antibodies to Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA), Hes-1 (a kind gift of Dr. Sudo, TORAY Corporation, Yokohama, Japan) [20], mouse monocyte-macrophage (MOMA2, Serotec, Oxford, UK), and ICAM-1 (Biolegend, San Diego, CA), was performed using standard procedures. Nuclei were counter-stained by methyl green

or 4',6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO). Macrophage accumulation was monitored by MOMA2 staining using a Vector ABC Biotin Kit and Vectastain DAB Substrate Kit (Vector Laboratories, Burlingame, CA). MOMA2-positive areas in atherosclerotic plaques were quantified with Win ROOF image analysis software (Win ROOF; Mitani, Fukui, Japan), using the average value for five locations from 10 mice for each group.

Detection of activated Notch1 in aortas

Protein samples from aortic lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were incubated with primary antibodies against cleaved Notch1 (Cell Signaling Technology, Beverly, MA), which denotes Notch1 receptor activated by γ -Secretase, and β -actin, (Sigma) followed by incubation with horseradish peroxidase (HRP)-conjugated anti-goat and anti-rabbit IgG antibodies (Santa Cruz), respectively. Antibody binding was visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

Flow cytometry

After the 8-week treatment, peripheral blood samples from 10 mice of the vehicle-and GSI-treated groups (LY411,575, 1.0 mg/kg/day) were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). The ratio of mature T cells to B cells in total lymphocytes was assessed by immunostaining with anti-CD3-FITC and anti-CD19-PE antibodies (BD Biosciences, Franklin Lakes, NJ), respectively. Events were analyzed using Cytomics FC-500 (Beckman Coulter, Fullerton, CA) and Beckman Coulter CXP software.

Histopathological analysis of atherosclerotic lesions

The top half of the hearts with 1 mm of proximal aorta attached was snap-frozen in OCT compound. From the area of the aortic valve leaflets, five to six serial 6- μ m sections were obtained and stained with H&E, oil red O, and Masson's trichrome to detect cellular morphology, lipids, and collagen accumulation, respectively. The atherosclerotic plaque area of the aortic sinus was assessed against the net area and the proportion of total intimal plaque lesion area to total cross-sectional vessel wall area, as defined by the external elastic lamina [21]. Quantitative image analysis was performed with Win ROOF.

ICAM-1 expression in primary cultured macrophages

Peritoneal macrophages were collected from the peritoneal cavity of ApoE^{-/-} mice, followed by peritoneal injection of 4% thioglycolate. The isolated cells were incubated overnight in RPMI containing 10% fetal bovine serum. Adherent macrophages were then treated with 100 nM LY411,575 or vehicle alone [dimethyl sulfoxide (DMSO)] for 24 h. Following treatment, the macrophages were fixed and immunostained for Hes-1 and ICAM-1 expression.

γ -Secretase inhibition in macrophages

RAW 264.7 cells (RIKEN, Japan) were treated with GSIs (LY411,575 at 100 nM and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *tert*-butyl ester or DAPT at 20 μ M) for 24 h. Cells were then lysed and the expressions of cleaved Notch1, ICAM-1, and β -actin were analyzed by Western blotting.

Migration assay

Migration of RAW 264.7 cells was estimated in a modified 48-well Boyden chamber (Neuro Probe) as described previously [6]. Cell suspensions (5×10^4 cells) were placed in the upper chamber with various concentrations of LY411,575 (0, 100, and 1 μ M). The lower chamber contained the same media with or without 10% fetal calf serum. The number of migrated cells

(mm⁻²) was estimated 6 h later. Experiments were performed in triplicate eight separate times for each group.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Differences were compared by one-way ANOVA followed by Fisher's test, and considered significant at $P < 0.05$. Frequencies were analyzed with the chi-squared test.

Results

Expression of Notch-related genes in aortas of ApoE^{-/-} mice

Real-time RT-PCR analysis showed that expression of Notch1 and a downstream transcriptional factor, Hes-1, was significantly increased up to approximately 3.7-fold in aortas from the ApoE^{-/-} mice compared to wild types (wild type vs. ApoE^{-/-}; Notch1, 100 ± 3.2 vs. $368 \pm 15\%$; Hes-1, 100 ± 3.8 vs. $369 \pm 10\%$; $n = 10$ in each group) (Fig. 1A). The expression levels of Notch3 and Notch ligand, Jagged-1, were also elevated significantly (wild type vs. ApoE^{-/-}; Notch3, 100 ± 1.6 vs. $216 \pm 8.9\%$; Jagged-1, 100 ± 3.8 vs. $274 \pm 7.0\%$; $n = 10$ in each; Fig. 1A). Notch1 and Hes-1 were also expressed in MOMA-2-positive cells in the plaque areas (Fig. 1B).

GSI suppresses Notch signaling activity of immune cells in atheromatous plaques

RT-PCR analysis also showed that Hes-1 expression decreased in the aortas of GSI-treated mice compared to those of vehicle-treated controls (Fig. 1A) (vehicle vs. GSI; $369 \pm 10\%$ vs. $138 \pm 11\%$, $P < 0.05$; $n = 10$ from each group). GSI treatment also decreased the expression of the Notch receptors and ligand (Fig. 1A) (vehicle vs. GSI; Notch1, $368 \pm 15\%$ vs. $163 \pm 13\%$; Notch3, $216 \pm 8.9\%$ vs. $118 \pm 8.9\%$; Jagged-1, $274 \pm 7.0\%$ vs. $166 \pm 5.7\%$; $P < 0.05$; $n = 10$). Immunohistochemical analysis showed virtually no Hes-1 expression in plaque-located macrophages in the GSI-treated mice (Fig. 1B). Indeed Notch1 receptor cleavage by γ -Secretase was significantly reduced in the aortas of GSI-treated mice (Fig. 1C). Notch activity in macrophages was thus augmented in the atherosclerotic plaques of ApoE^{-/-} mice, and this effect was abolished by the GSI treatment.

Systemic effects of GSI

Low-dose continuous administration of LY411,575 (0.2 and 1.0 mg/kg/day) for 8 weeks was not attributed to mortality, incidence of diarrhea (data not shown), body weight, or lipid profile (Table 1). Chronic treatment with a lower dose of GSI (0.2 mg/kg/day) did not alter the histology of the intestine and thymus (data not shown). However, administration of 1.0 mg/kg/day LY411,575 for 8 weeks increased goblet cells in the ileum and induced cortical atrophy of the thymus, consistent with a previous report (Fig. 2A) [16]. FACS analysis of peripheral blood also revealed lower numbers of mature T cells (CD3⁺) and B cells (CD19⁺) in these mice (Fig. 2B and C).

Effect of GSI on the progression of atherosclerosis in ApoE^{-/-} mice

Histological analysis showed that GSI treatment significantly reduced atherosclerotic lesion formation in a dose-dependent manner (vehicle, GSI at 0.2 and 1.0 mg/kg/day; 368 ± 19 , 301 ± 22 , $208 \pm 19 \times 10^3 \mu\text{m}^2$, respectively), revealed by 18.4 and 45.8% reductions in plaque area, respectively (vehicle vs. GSI at 0.2 mg/kg/day, $P < 0.03$; vehicle vs. GSI at 1.0 mg/kg/day, $P < 0.01$; $n = 10$ from each group; Fig. 3A and B). Plaque burden in GSI-treated mice was also markedly decreased in accordance with the localized plaque reduction [lipid deposit areas (% plaque areas); (vehicle, GSI at 0.2 mg/kg/day and 1.0 mg/kg/day; 44.8 ± 1.9 , 38.8 ± 1.9 , $27.7 \pm 1.5\%$, respectively) (vehicle vs. GSI at 0.2 mg/kg/day, $P < 0.03$; $n = 10$ from each group);

vehicle vs. GSI at 1.0 mg/kg/day, $P < 0.01$; $n = 10$ from each group; Fig. 3A and C]. There was no detectable difference in collagen accumulation among the three groups based on the Masson's trichrome staining (Fig. 3A and data not shown).

Effect of GSI on macrophage accumulation

There were significantly fewer macrophages in plaques in GSI-treated mice, compared to vehicle-treated controls (MOMA2-positive area; vehicle vs. GSI; $35.1 \pm 1.3\%$ vs. $25.2 \pm 1.2\%$ of plaque areas; $P < 0.01$; $n = 10$ from each group; Fig. 4A). As Notch signaling augments macrophage activity with a concomitant increase in ICAM-1, we tested how GSI treatment affected ICAM-1 expression in isolated macrophages. GSI treatment reduced ICAM-1 expression in primary-cultured peritoneal macrophages, and the change was in proportion to the reduced Hes-1 expression (Fig. 4B). We also examined the effects of two kinds of GSIs on RAW 264.7 cells. γ -Secretase inhibition with either DAPT (20 μ M) or LY411,575 (100 nM) decreased Notch1 cleavage, resulting in reduced expression of ICAM-1 (Fig. 4C).

Finally, a modified Boyden chamber assay was performed to assess whether the GSI treatment suppressed the migratory activity of a macrophage cell line, RAW264.7. GSI treatment also suppressed macrophage migratory activity in response to serum in proportion to the concentrations of LY411,575 (Fig. 4D). Thus, Notch inhibition by GSI treatment suppressed macrophage migration, suggesting a reduction of plaque in GSI-treated atheromatous lesions.

Discussion

The present study demonstrated that the γ -Secretase inhibitor, LY411,575, significantly attenuated the development of atherosclerotic lesions in apolipoprotein E-deficient mice. This effect was associated with a significant suppression of Notch signaling activity in macrophages in atheromatous plaques. Together, these findings indicated that γ -Secretase inhibition *in vivo* reduces the accumulation of macrophages in atherogenic areas. Indeed, the GSI treatment reduced the macrophage expression of ICAM-1, which regulates migratory activity. Notch signal inhibition with GSI thus seems to suppress macrophage activity in atherosclerotic lesions and thereby could reduce plaque formation.

Macrophage recruitment to atherosclerotic plaques is critical for the local inflammatory response, and results in reduced plaque stability [1]. The regulation of inflammatory cells in plaques is therefore a novel therapeutic target to potentially inhibit the progression of atherosclerosis [3]. Notch signaling alters not only the fate specification of immature lymphocytes and myeloid cells [7,9,22], but also macrophage function [10], and overexpression of Notch receptors and ligands was implicated in the inflammation associated with atheromatous plaques [11,12]. Indeed, the aortas of ApoE^{-/-} mice in this study showed upregulated expression of Notch-related molecules, and macrophages in the plaques were strongly immunopositive for Notch1 and downstream transcriptional factor Hes-1. These findings collectively suggest that the Notch signaling pathway is activated in the process of atherosclerotic plaque development.

The present study further indicated that GSI treatment of ApoE^{-/-} mice suppressed Notch signaling activity in the atheromatous plaques. The treatment also efficiently reduced Notch1 cleavage, resulting in decreased Hes-1 expression in macrophages. The γ -Secretase inhibition similarly reduced the expression of Hes-1 and adhesion molecule, ICAM-1, as observed previously in an *in vitro* study [10]. The migratory activity of RAW 264.7 macrophages also decreased in proportion to the GSI dose. Since ICAM-1 is required for macrophage migration in atheromatous plaques [23], Notch signal inhibition with GSIs should decrease accumulation of macrophages in plaques and the plaque burden in ApoE^{-/-} mice.

Inhibition of Notch signaling with GSI treatment was recently shown to decrease monocyte infiltration and ICAM-1 expression in ischemic brain tissue, with a resultant decrease in brain damage followed by ischemia-reperfusion injury [16]. Thus, Notch pathway inhibition could be physiologically relevant in certain inflammatory diseases. In this study, we showed that long-term administration of GSI reduced the progression of atherosclerotic plaques in ApoE^{-/-} mice by altering the immune response. The Notch signaling system is therefore a potential target for modulating abnormal immune responses such as for inflamed atheromatous plaques, and inhibition of Notch signaling with GSIs might serve as a novel anti-inflammatory therapy [24].

To address the therapeutic window of LY411, 575, the present study also tested two low oral doses (0.2 and 1.0 mg/kg/day), and carefully monitored the mice for typical symptoms of adverse effects according to previous reports [15,18]. Higher doses of LY411,575 (10 mg/kg/day for 6 days) have been implicated in loss of body weight and gastrointestinal toxicity [18, 25]. No mice in the present study showed serious side effects such as death or malnutrition due to toxicity after 8-week administration of the lower dose. There was also no increase in goblet cells in the ileum as an indicator of less severe effects. This accords with a previous report of no change in goblet cells in the ileum after 20 days of LY411,575 at 1.0 mg/kg/day [18], although this higher dose did induce increased goblet cells in the ileum after the 8-week administration in this study. Thus, long-term administration of GSIs at a low dose would seem to integrate any adverse effects.

The present study has certain limitations. The GSI treatment could also affect the activity of additional type I transmembrane receptors (i.e., amyloid precursor protein, Erb-4, CD44, E-, and N-cadherin), which are potential substrates for γ -Secretase [26]. Therefore, it was not possible to associate the observed effects with inhibition of Notch signaling, or ascertain the specific Notch homologs involved in atherogenesis.

In conclusion, our findings clearly demonstrated that pharmacological inhibition of γ -Secretase suppresses Notch signaling in immune cells of atherosclerotic lesions, resulting in a reduced inflammatory response and plaque formation. GSI treatment could be potentially beneficial in patients with atherosclerotic burden if the possible adverse side effects can be avoided using an efficient drug delivery system.

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Fig. 1.

(A) Semiquantitative analysis of expression of Notch-related molecules ($n = 10$ from each group). Values were normalized to β -actin expression. Results are presented as mean $\pm$ SEM of 10 mice. * $P < 0.05$ compared with wild-type mice. † $P < 0.05$ compared with vehicle-treated ApoE^{-/-} mice. (B) Expression of Notch1 and Hes-1 in macrophages in atherosclerotic plaques. MOMA2-positive cells (Red) in plaques of control mice show highly expressed Notch1 and Hes-1 (green), unlike those of GSI-treated mice. Scale bar = 100 μ m. (C) Expression of cleaved Notch1 and β -actin (internal control) in aortas of ApoE^{-/-} mice after vehicle or GSI treatment was determined by immunoblot analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Systemic effects of GSI. (A) Representative images of intestine and thymus from vehicle- and GSI-treated ApoE^{-/-} mice. Intestine of GSI-treated mice showed increased goblet cells (dark violet cells) compared to vehicle-treated mice. Thymus of GSI-treated mice showed severe cortical atrophy. Co: cortex, Ma: marrow. (B) and (C) GSI treatment decreased the numbers of mature T cells (CD3⁺) (B) and B cells (CD19⁺) (C). Results are mean ± SEM. *n* = 10 for each group. **P* < 0.01, compared with vehicle-treated mice). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

GSI reduces plaque formation in ApoE^{-/-} mice. (A) Representative images showing aortic cusps stained with hematoxylin-eosin (top), Masson's trichrome (middle), and oil red O (bottom). Left and right panels show aortic cusps from vehicle-treated and GSI-treated ApoE^{-/-} mice, respectively. Scale bar = 300 μm . (B) Plaque areas of aortic cusps ($*P < 0.03$, $\#P < 0.01$, $n = 10$, compared with vehicle-treated mice, respectively). (C) Lipid content in plaque areas of aortic cusps (oil red O-positive areas) ($*P < 0.03$, $\#P < 0.01$, $n = 10$, compared with vehicle-treated mice, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

GSI treatment decreases macrophage accumulation and activity. (A) Representative micrographs of immunohistochemistry for MOMA2 (left) expression. MOMA2-positive areas were significantly less in GSI-treated mice compared to control mice. Results are mean $\pm$ SEM. $n = 10$ for each group. (* $P < 0.01$; right). (B) Immunohistochemistry for ICAM-1 and Hes-1 in primary-cultured peritoneal macrophages. (C) Expression of cleaved Notch1, ICAM-1, and β -actin (internal control) in RAW 264.7 cells after treatment with control or GSI was determined by immunoblot analysis. (D) Migration assay in presence or absence of GSI. Results are mean $\pm$ SEM. $n = 8$ for each group. (* $P < 0.005$, # $P < 0.001$, compared with 0 nM with 10% serum, respectively).

Table 1

Characteristics of GSI-treated ApoE-deficient mice fed Western diet for 2 months.

	Vehicle	GSI		P Value
		0.2 mg/kg/day	1.0 mg/kg/day	
<i>Before treatment</i>				
Body weight (g)	20.5 ± 1.9	20.4 ± 1.9	20.4 ± 2.0	NS
<i>After treatment</i>				
Body weight (g)	27.9 ± 1.6	27.0 ± 2.1	26.7 ± 2.3	NS
TC (mg/dl)	1319 ± 257	1291 ± 288	1210 ± 312	NS
TG (mg/dl)	236 ± 115	240 ± 110	209 ± 107	NS
HDL-C (mg/dl)	21 ± 10	22 ± 11	25 ± 11	NS