Oral administration of the β-glucan produced by *Aureobasidium pullulans* ameliorates development of atherosclerosis in apolipoprotein E deficient mice.

Shiho Aoki [a], Atsushi Iwai [a, b*], Koji Kawata [a, b], Daisuke Muramatsu [a], Hirofumi Uchiyama [b], Mitsuyasu Okabe [b], Masahiro Ikesue [c], Naoyoshi Maeda [c], and Toshimitsu Uede [c, d].

[a] Aureo Science Co., Ltd., Sapporo, Hokkaido, Japan; [b] Aureo Co., Ltd., Kimitsu, Chiba, Japan; [c] Division of Molecular Immunology and [d] Department of Matrix 3Medicine, Institute for Genetic Medicine, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan.

*** Correspondence should be addressed to Atsushi Iwai, Aureo Science Co., Ltd., Hokudai Business Spring, North 21, West 12, Kita-ku, Sapporo, Hokkaido, 001-0021, Japan; Phone: +81-11-757-1316; Fax: +81-11-738-1317; E-Mail: iwai-atsushi@aureo.co.jp**

Abstract

The *Aureobasidium pullulans*-produced β-glucan (AP-PG) is an immune stimulator, and believed to exhibit beneficial effects on health through its immune stimulating activity. Here, the effect of oral administration of AP-PG on high-fat diet (HFD)-induced atherosclerosis was evaluated using apolipoprotein E deficient mice, a widely used mouse model for atherosclerosis. The results demonstrated that HFD-induced development of atherosclerosis was significantly reduced in the AP-PG-treated mice when compared with that of the control mice. In serological analysis, blood levels of oxidized low-density lipoprotein cholesterol, a well-known risk factor for the development of atherosclerosis, were significantly reduced in the AP-PG-treated group of mice. Further, immunohistochemical analysis using MOMA-2 antibody showed that oral administration of AP-PG is effective in ameliorating vascular accumulation of macrophages. These data suggest the possibility that oral administration of AP-PG is effective in ameliorating HFD-induced development of atherosclerosis.

Key words: β-glucan, *Aureobasidium pullulans*, atherosclerosis

Abbreviations: ApoE, Apolipoprotein E; AP-PG, *A. pullulans*-produced β-glucan;

CAD, coronary artery disease; HDL, high-density lipoprotein; HFD, high-fat diet; LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein

1. Introduction

Black yeast, *Aureobasidium pullulans*, extracellularly produces a soluble β-glucan (AP-PG) consisting of a β-(1,3)-linked main chain and β-(1,6)-branched glucose residues under certain conditions (Moriya et al., 2013), and the β-glucan-containing *A. pullulans*-cultured fluid is consumed as a supplemental food promoting health. The *A. pullulans*-produced β-glucan is highly branched with β-(1,6)-linked glucose residues, and is known to be structurally distinct from other organism derived β-glucans. It is believed that the *A. pullulans*-produced β-glucan is effective in promoting health, and has anti-tumour (Kataoka-Shirasugi, Ikuta, Kuroshima, & Misaki, 1994; Kimura, Sumiyoshi, Suzuki, & Sakanaka, 2006), anti-allergy (Kimura, Sumiyoshi, Suzuki, Suzuki, & Sakanaka, 2007), and anti-infectious disease (Yatawara et al., 2009; Muramatsu et al., 2012; Muramatsu et al., 2014) effects.

In this study, we demonstrated that oral administration of AP-PG is effective in ameliorating HFD-induced development of atherosclerotic lesions in apolipoprotein E (ApoE) deficient mice which spontaneously develop atherosclerotic lesions (Zhang, Reddick, Piedrahita, & Maeda, 1992; Plump et al., 1992). In the serological analysis, the blood level of oxidized LDL cholesterol increased after feeding with HFD, and this increment was significantly decreased in AP-PG administered mice compared with that in the control mice. These results suggest that oral administration of AP-PG is effective in preventing HFD-induced development of atherosclerosis.

2. Material and methods

2.1. Mice

Specific pathogen-free 8 week old ApoE deficient male mice of C57BL/6N background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were performed in accordance with the guidelines of the Bioscience Committee of Hokkaido University and were approved by the Animal Care and Use Committee of Hokkaido University (permit number 13-0179).

2.2. Preparation of *A. pullulans*-derived β-glucan

For preparation of the *A. pullulans*-derived β-glucan (AP-PG), *A. pullulans*-cultured fluid was subjected to diatomaceous earth filtration to remove cell debris, and this was

followed by removal of low molecular weight components by ultrafiltration with a cut-off molecular weight of 20,000 Da (Q2000; Advantec, Tokyo, Japan). Subsequently, β-glucan was precipitated with ethanol at the concentration of 80 %, and used in this study. The purity of the prepared β-glucan was estimated to be above 99% using high performance liquid chromatography (HPLC). The low molecular weight fraction enriched by ultrafiltration was used as the control in this study. The removal of β-glucan from the control fraction was confirmed using HPLC, and the concentration of β-glucan was estimated to be below 1%.

2.3. Measurement of cholesterols and triacylglycerol

Serum concentrations of cholesterol and triacylglycerol were measured using commercially available kits (Triglyceride E-test Kit and Cholesterol E-test Kit; Wako Pure Chemical, Osaka, Japan). Blood levels of high-density lipoprotein (HDL) cholesterol and oxidized low-density lipoprotein (ox-LDL) were monitored using the HDL cholesterol E-test Wako (Wako) and an ELISA Kit for Oxidized Low Density Lipoprotein (USCN Life Science Inc, Wuhan, China), respectively. Each procedure was performed according to the manufacturer's protocols, and the blood low-density lipoprotein cholesterol levels were calculated by the following formula: LDL cholesterol = total cholesterol – HDL cholesterol – (triacylglycerol x 0.2).

2.4. Histochemical and immunohistochemical analysis

The MOMA-2 antibody was purchased from Serotec, Oxford, UK and Sigma-Aldrich, St. Louis, MO, USA. The procedure for the immunochitochemical staining, haematoxylin and eosin (HE) staining, Oil-O -Red staining, and immunohistochemical staining are described elsewhere (Matsui et al., 2003).

2.5. Image analysis and statistical analysis

The analysis of the histochemical images was performed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). To check for significant differences between the indicated pairs of data, the two-tailed unpaired Student's t-test was used in this study.

3. Results

3.1. Oral administration of AP-PG is effective to ameliorate development of atherosclerosis in ApoE deficient mice.

To evaluate the effects of orally administered AP-PG to HFD-induced

arteriosclerosis, we used ApoE deficient mice. An outline of the experimental design is shown in Figure 1 The AP-PG in the drinking water was diluted to a concentration of 200 μg/ml, and the AP-PG dose under this condition was estimated to be about 2 mg/kg as evaluated by the drinking water intake. After one week of pretreatment with AP-PG, the mice were given the HFD for 16 weeks, and the administration of AP-PG was continued till the end of the experiment. The body weight and food intake changes of the mice are shown in Supplemental Figure S1. The results show that the oral administration of AP-PG did not significantly affect the food intake and body weight of the mice.

Next, the effect of orally administered AP-PG on the HFD-induced development of atherosclerosis was assessed by Oil-Red-O staining. The image analysis and microphotographs of Oil-Red-O–stained aortae are shown in Figures 2A and 2B, respectively. These results show that Oil-Red-O stained aortic atherosclerosis was significantly augmented by the HFD in the control group mice; the HFD-induced aortic atherosclerosis was significantly reduced in the AP-PG administered group of mice. We also evaluated the development of atherosclerotic lesions around the aortic roots. The HFD induced-atherosclerosis around the aortic roots was significantly lower in the AP-PG administered mice than in the control mice (Figure 2C and 2D). These results indicate that the oral administration of AP-PG is effective to ameliorate HFD-induced development of atherosclerosis in ApoE deficient mice.

3.2. Oral administration of AP-PG reduces blood levels of oxidized low-density lipoprotein cholesterol.

To evaluate effects of orally administered AP-PG on the lipid metabolism, the blood levels of cholesterol and triacylglycerol were measured using commercially available kits. The results show that blood cholesterol and triacylglycerol levels were not significantly different for the HFD AP-PG and HFD control groups (Figure 3A and 3B, respectively). Next, the blood levels of high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol were quantified. The results show that the amounts of blood HDL and LDL cholesterols were also not significantly changed by the oral administration of AP-PG (Figure 3C and 3D, respectively). Finally, the blood level of oxidized LDL (ox-LDL) cholesterol was measured. The results show that the blood ox-LDL cholesterol level was significantly reduced in the HFD AP-PG group mice, compared with that in the HFD control group (Figure 3E).

3.3. Macrophage accumulation in vascular walls is reduced by oral administration

of AP-PG

To evaluate the effects of orally administered AP-PG on macrophage accumulation and foam cell formation, sections of aortic roots were immunohistochemically analyzed using MOMA-2 antibody. The image analysis results and histological microphotographs are shown in Figures 4A and 4B, respectively. These results show that the macrophage accumulation as defined by the MOMA-2 positive areas were significantly smaller in the HFD AP-PG group mice than in the HFD control group mice (Figure 4A and 4B). The data suggest that oral administration of AP-PG is effective to ameliorate vascular accumulation of macrophages and foam cell formation from the macrophages.

4. Discussion

This study demonstrates that oral administration of AP-PG is effective to ameliorate the development of atherosclerosis caused by HFD in ApoE deficient mice. Serological analysis indicated that the blood ox-LDL level was significantly lower in the AP-PG group mice than in the control group mice (Figure 3E). The blood ox-LDL level is closely related to the development of atherosclerosis (Itabe & Ueda, 2007; Mitra, Deshmukh, Sachdeva, Lu, & Mehta, 2011), and the reduction of blood ox-LDL level after oral administration of AP-PG could be hypothesized to be an important mechanism for the amelioration of HFD-induced arteriosclerosis.

In this study, to evaluate the effects of the β-glucan in *A. pullulans*-cultured fluid clearly, purified β-glucan (AP-PG) was prepared, and a β-glucan-free fraction of *A. pullans*-cultured fluid was used as the control. Although this fraction was assumed to be containing several low molecular weight components, the body weight changes of control group mice were almost the same as that of the mice received with water without additive (data not shown). Therefore, administration of the β-glucan-free fraction is not thought to significantly affect the results in this study.

β-glucans including AP-PG are known to be a dietary fibre, and are not absorbed in human and mouse bodies. On the other hand, the results of our preliminary experiments using radiolabeled cholesterol indicate that oral administration of AP-PG does not significantly inhibit cholesterol absorption in the small intestine under the experimental condition of this study (Aoki et al., 2015). Therefore, the influence of AP-PG as a dietary fibre on the results shown in this study is assumed to be weak, and other effects of AP-PG is thought to be important in ameliorating HFD-induced development of atherosclerosis.

Inflammation followed by oxidative stress caused by reactive oxygen species (ROS) production is closely related to ox-LDL production. Here it should be noted that

there are reports of anti-inflammatory effects of β-glucan produced by *A. pullulans* (Kimura, Sumiyoshi, Suzuki, Suzuki, & Sakanaka, 2007; Tanaka, Tanaka, Suzuki, & Mizushima, 2011). In addition, several reports have demonstrated antioxidative effects of β-glucans derived from other organisms (Mirjanaa et al., 2013; Uskoković et al., 2013; Suchecka et al., 2015). Therefore, it is tempting to speculate that AP-PG has antioxidative effects, and that the anti-inflammatory and antioxidative effects of AP-PG may be involved in the reduction of blood ox-LDL levels.

The results of the immunohistochemical analysis indicated that MOMA-2 positive areas were reduced by the oral administration of AP-PG (Figure 4), suggesting that vascular accumulation of macrophages and subsequent foam cell formation were reduced by oral administration of AP-PG. Since vascular accumulation of macrophages is closely related to the blood ox-LDL cholesterol levels (Maiolino et al., 2013; Yu, Fu, Zhang, Yin, & Tang, 2013), the reduction of macrophage numbers in the vascular walls may be due to the reduction of blood ox-LDL cholesterol levels by the oral administration of AP-PG. A previous report demonstrated that orally adiministered β-glucan was able to activate lymphocytes localizing Peyer's patch (Hashimoto, Suzuki, & Yadomae, 1991). Thus, the immune modulating activity of AP-PG is thought to be expressed by an indirect manner through the activation of small intestinal immunity. In addition, there is a report of the modulating effects of oral administration of *Sclerotinia sclerotiorum*-derived β-glucan on alveolar macrophage functions (Sakurai et al., 1992), suggesting the possibility that orally administered AP-PG modulates the immune response of circulating macrophages, a source of vascular wall macrophages.

5. Conclusions

In the present study, we demonstrated that orally administered AP-PG induced amelioration of HFD-induced atherosclerosis in a mouse model. Also, *A. pullulans*-cultured fluid containing β-glucan as a main component is commonly consumed as a supplemental food for long periods, and the safety as a food additive is well established. In addition, there were no negative effects on the AP-PG administered mice in this study, supporting the safety of AP-PG. However, further study is needed to establish whether AP-PG as a supplement has beneficial effect on the development of atherosclerosis in humans.

Appendix: Supplementary material

Supplementary data to this article can be found in an attached file.

References

Aoki, S., Iwai, A., Kawata, K., Muramatsu, D., Uchiyama, H., Okabe, M., Ikesue, M., Maeda, N. & Uede, T. (2015). Oral administration of the *Aureobasidium pullulans*-derived β-glucan effectively prevents the development of high fat diet-induced fatty liver in mice. *Sci Rep* In press. doi: 10.1038/srep10457

Hashimoto, K., Suzuki, I., & Yadomae, T. (1991). Oral administration of SSG, a beta-glucan obtained from Sclerotinia sclerotiorum, affects the function of Peyer's patch cells. *Int J Immunopharmacol*, 13(4), 437-442.

Itabe, H. & Ueda, M. (2007). Measurement of plasma oxidized low-density lipoprotein and its clinical implications. *J Atheroscler Thromb* 14, 1-11.

Kataoka-Shirasugi, N., Ikuta, J., Kuroshima, A., & Misaki, A. (1994). Antitumor activities and immunochemical properties of the cell-wall polysaccharides from *Aureobasidium pullulans*. *Biosci Biotechnol Biochem*, 58(12), 2145-2151.

Kimura, Y., Sumiyoshi, M., Suzuki, T., & Sakanaka, M. (2006). Antitumor and antimetastatic activity of a novel water-soluble low molecular weight β-1, 3-D-glucan (branch β-1,6) isolated from *Aureobasidium pullulans* 1A1 strain black yeast. *Anticancer Res*, 26(6B), 4131-4141.

Kimura, Y., Sumiyoshi, M., Suzuki, T., Suzuki, T., & Sakanaka, M. (2007). Inhibitory effects of water-soluble low-molecular-weight β-(1,3-1,6) d-glucan purified from *Aureobasidium pullulans* GM-NH-1A1 strain on food allergic reactions in mice. *Int Immunopharmacol*, 7(7), 963-972.

Maiolino, G., Rossitto, G., Caielli, P., Bisogni, V., Rossi, G. P., & Calo, L. A. (2013). The role of oxidized low-density lipoproteins in atherosclerosis: the myths and the facts. *Mediators Inflamm*, 2013, 714653.

Matsui, Y., Rittling, S. R., Okamoto, H., Inobe, M., Jia, N., Shimizu, T. (2003). Osteopontin deficiency attenuates atherosclerosis in female apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*, 23(6), 1029-1034.

Mirjanaa, M., Jelenaa, A., Aleksandraa, U., Svetlanaa, D., Nevenaa, G., Jelenaa, M. (2013). β-Glucan administration to diabetic rats reestablishes redox balance and stimulates cellular pro-survival mechanisms. Journal of Functional Foods, **5**(1), 267-278.

Mitra, S., Deshmukh, A., Sachdeva, R., Lu, J., & Mehta, J. L. (2011). Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. *Am J Med Sci, 342*(2), 135-142.

Moriya, N., Moriya, Y., Nomura, H., Kusano, K., Asada, Y., Uchiyama, H. (2013).

Improved beta-glucan yield using an Aureobasidium pullulans M-2 mutant strain in a 200-L pilot scale fermentor targeting industrial mass production. *Biotechnol. Bioprocess Eng., 18*(6), 1083-1089.

Muramatsu, D., Iwai, A., Aoki, S., Uchiyama, H., Kawata, K., Nakayama, Y. (2012). β-Glucan derived from *Aureobasidium pullulans* is effective for the prevention of influenza in mice. *PLoS One, 7*(7), e41399.

Muramatsu, D., Kawata, K., Aoki, S., Uchiyama, H., Okabe, M., Miyazaki, T. (2014). Stimulation with the *Aureobasidium pullulans*-produced β-glucan effectively induces interferon stimulated genes in macrophage-like cell lines. *Sci Rep, 4*, 4777.

Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J. G. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell, 71*(2), 343-353.

Sakurai, T., Hashimoto, K., Suzuki, I., Ohno, N., Oikawa, S., Masuda, A. (1992). Enhancement of murine alveolar macrophage functions by orally administered β-glucan. *Int J Immunopharmacol, 14*(5), 821-830.

Suchecka, D., Harasym, J. P., Wilczak, J., Gajewska, M., Oczkowski, M., Gudej, S. (2015). Antioxidative and anti-inflammatory effects of high β-glucan concentration purified aqueous extract from oat in experimental model of LPS-induced chronic enteritis. Journal of Functional Foods, 14(0), 244-254.

Tanaka, K., Tanaka, Y., Suzuki, T., & Mizushima, T. (2011). Protective effect of β-(1,3 --> 1,6)-D-glucan against irritant-induced gastric lesions. *Br J Nutr, 106*(4), 475-485.

Uskoković, A., Mihailović, M., Dinić, S., Jovanović, J. A., Grdović, N., Marković, J. (2013). Administration of a β-glucan-enriched extract activates beneficial hepatic antioxidant and anti-inflammatory mechanisms in streptozotocin-induced diabetic rats. *Journal of Functional Foods, 5*(4), 1966-1974.

Yatawara, L., Wickramasinghe, S., Nagataki, M., Takamoto, M., Nomura, H., Ikeue, Y. (2009). *Aureobasidium*-derived soluble branched (1,3-1,6) β-glucan (Sophy β-glucan) enhances natural killer activity in Leishmania amazonensis-infected mice. *Korean J Parasitol, 47*(4), 345-351.

Yu, X. H., Fu, Y. C., Zhang, D. W., Yin, K., & Tang, C. K. (2013). Foam cells in atherosclerosis. *Clin Chim Acta, 424*, 245-252.

Zhang, S. H., Reddick, R. L., Piedrahita, J. A., & Maeda, N. (1992). Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science, 258*(5081), 468-471.

Figure legends

Figure 1. Experimental design of this study

Figure 2. Oral administration of AP-PG effectively prevented high-fat diet-induced development of atherosclerotic plaque in ApoE deficient mice.

(A, B) At the end of the period of the experiment, the main artery was isolated from the mice and atherosclerotic plaque formation was evaluated using Oil-O-Red staining. Data representing the image analysis results **(A)** and histopathological specimen images **(B)**. Scale bars indicate 1 cm. **(C)** Image analysis of the atherosclerotic lesions on the aortic root sections. **(D)** Microphotographs of the histology of the aortic root sections stained with Oil-O-Red. Scale bars indicate 50 μm. Error bars indicate the standard deviation. NC: normal control diet. HFD: high-fat diet. n.s.: not significant.

Figure 3. Evaluation of the effects of orally administered AP-PG on blood cholesterol levels

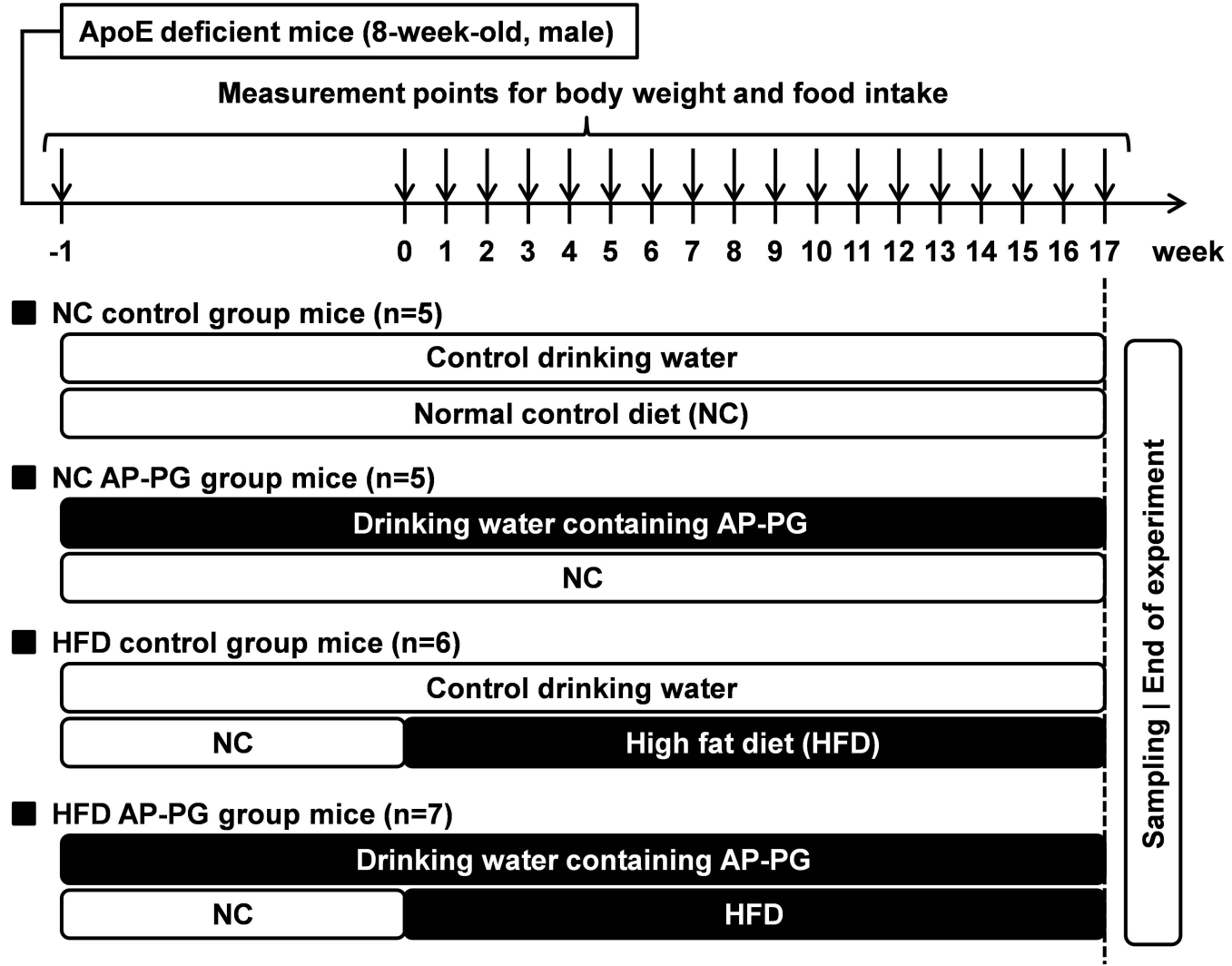
(A-E) At the end of the period of the experiment, plasma levels of total cholesterol **(A)**, triacylglycerol **(B)**, high-density lipoprotein (HDL) cholesterol **(C)**, low-density lipoprotein (LDL) cholesterol **(D)**, and oxidized low-density lipoprotein (ox-LDL) cholesterol **(E)** in the mice were measured as described in Methods section. Error bars indicate m of standard deviation. NC: normal control diet. HFD: high-fat diet. n.s.: not significant.

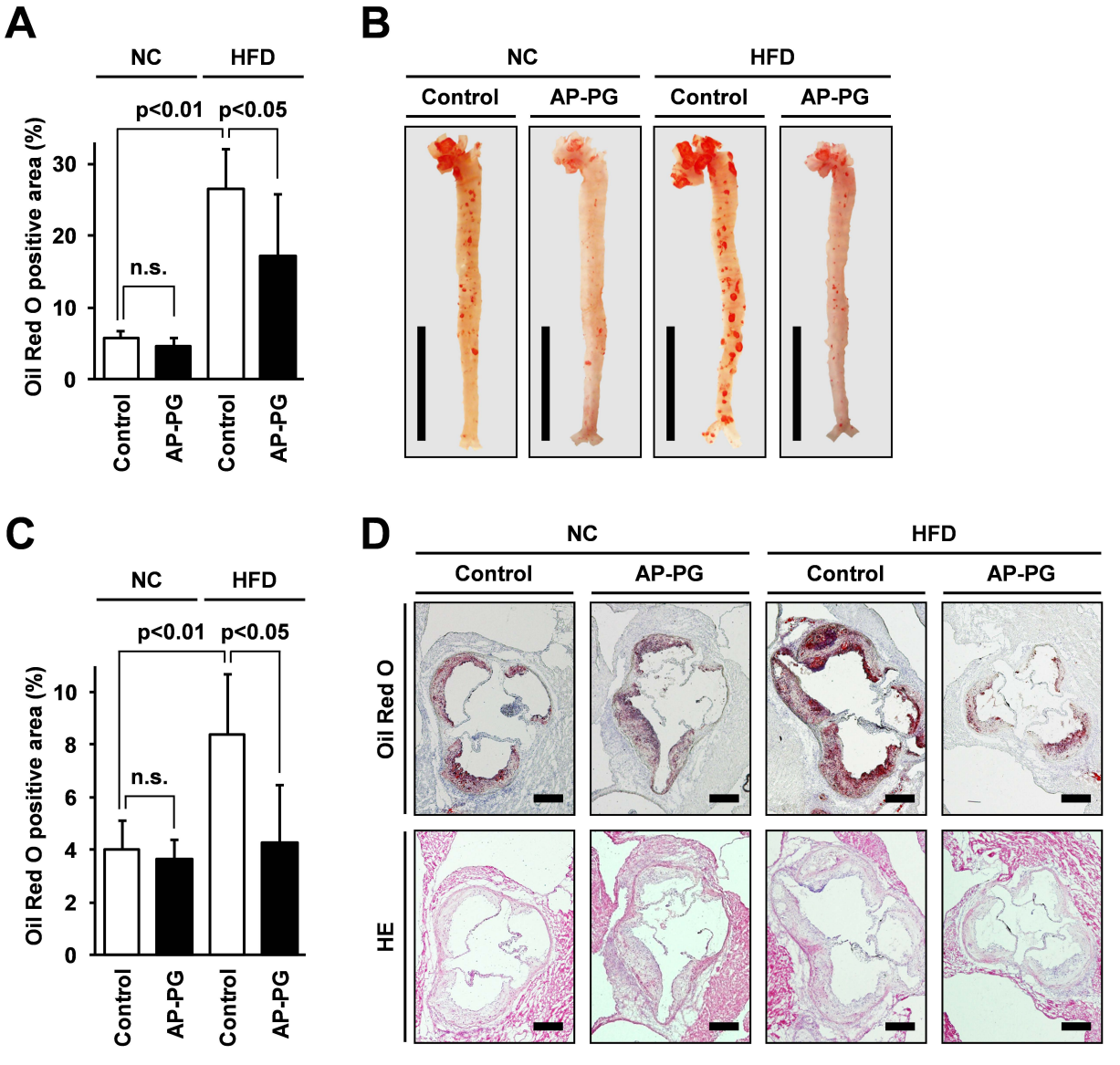
Figure 4. Oral administration of AP-PG is effective to prevent vascular accumulation of macrophages.

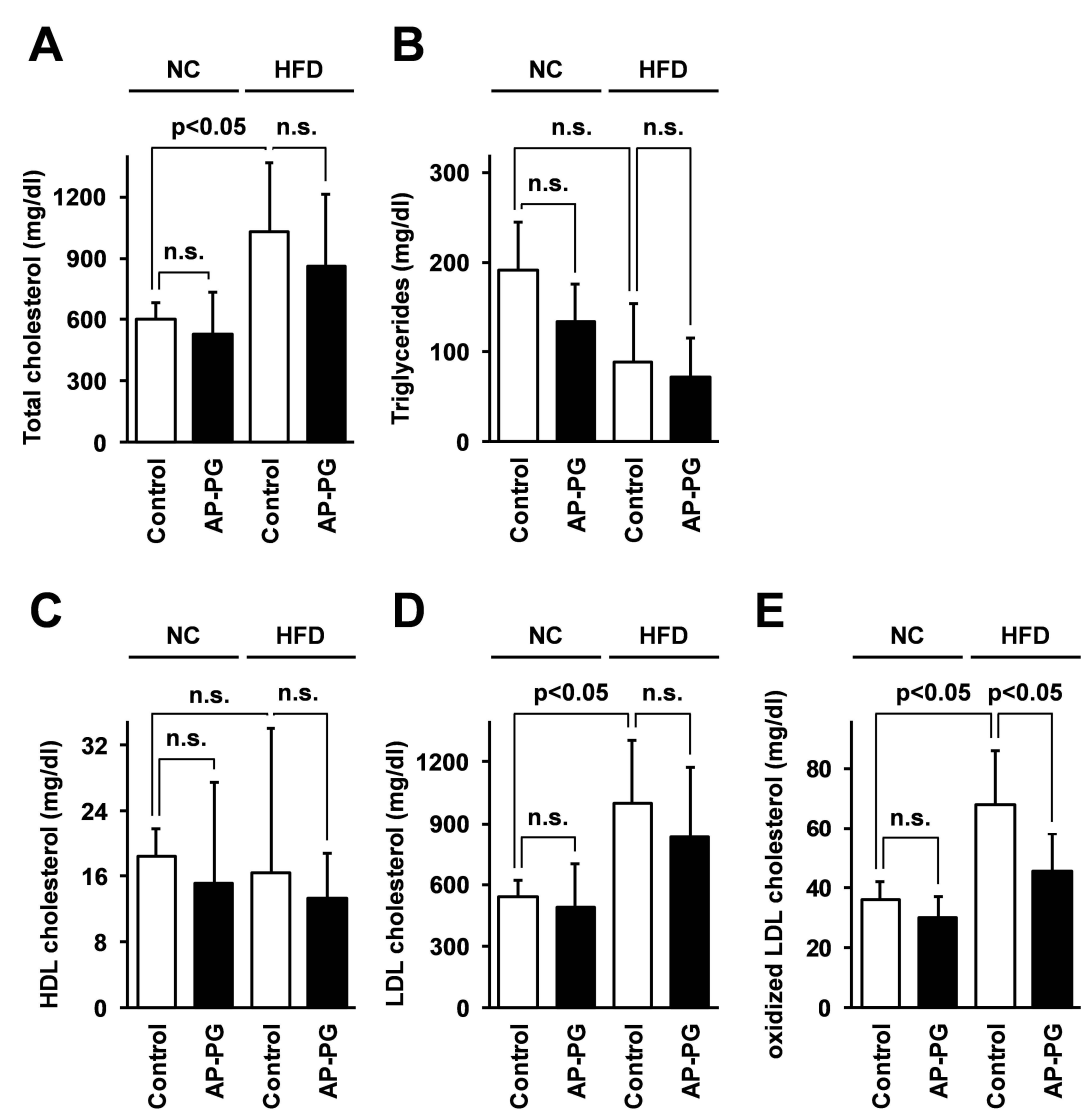
(A, B) Sections of aortic roots sampled from the mice were immunostained with MOMA-2. **(A)** Percentages of MOMA-2 positive areas were calculated by image analysis. Error bars indicate the standard deviation. NC: normal control diet. HFD: high-fat diet. n.s.: not significant. **(B)** Microphotographs of immunohistology sections. Scale bars indicate 300 μm.

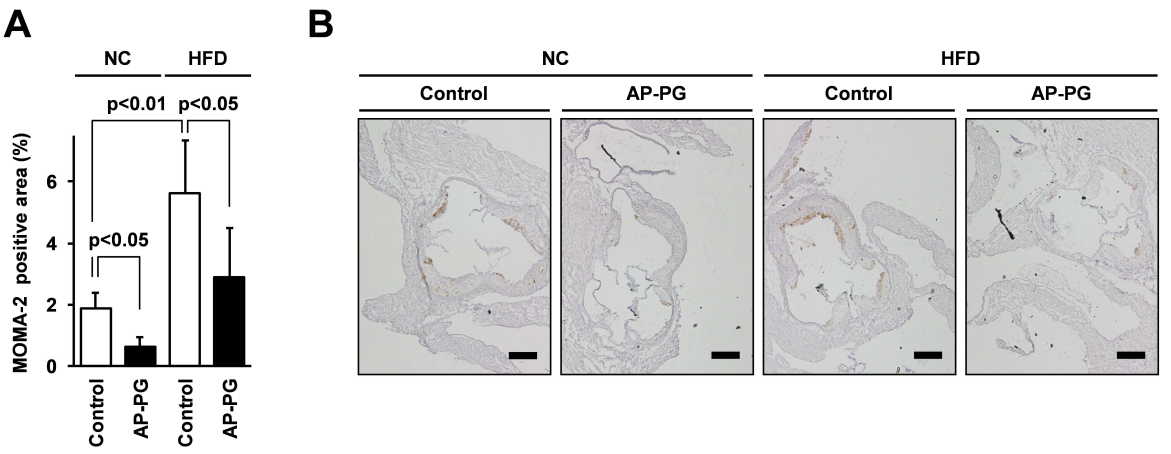
Highlights

***Aureobasidium pullulans*-derived β-glucan (AP-PG) is known to be an immunomodulator.**
Apolipoprotein E deficient (ApoE) mice spontaneously develop atherosclerosis.
AP-PG is effective in ameliorating development of atherosclerosis in the model mice.
AP-PG administration reduces oxidized low-density lipoprotein cholesterol levels.
Oral administration of AP-PG ameliorates vascular accumulation of macrophages.


ApoE deficient mice (8-week-old, male)
Measurement points for body weight and food intake
-1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
week
NC control group mice (n=5)
Control drinking water
Normal control diet (NC)
NC AP-PG group mice (n=5)
Drinking water containing AP-PG
NC
HFD control group mice (n=6)
Control drinking water
NC
High fat diet (HFD)
HFD AP-PG group mice (n=7)
Drinking water containing AP-PG
NC
HFD
Sampling | End of experiment


A
NC
HFD
p<0.01
p<0.05
n.s.
Oil Red O positive area (%)
30
20
10
0
Control
AP-PG
Control
AP-PG
B
NC
HFD
Control
AP-PG
Control
AP-PG
C
NC
HFD
p<0.01
p<0.05
n.s.
Oil Red O positive area (%)
10
8
6
4
2
0
Control
AP-PG
Control
AP-PG
D
NC
HFD
Control
AP-PG
Control
AP-PG
Oil Red O
HE


A
NC
HFD
p<0.05
n.s.
n.s.
Total cholesterol (mg/dl)
1200
900
600
300
0
Control
AP-PG
Control
AP-PG
B
NC
HFD
n.s.
n.s.
n.s.
Triglycerides (mg/dl)
300
200
100
0
Control
AP-PG
Control
AP-PG
C
NC
HFD
n.s.
n.s.
n.s.
HDL cholesterol (mg/dl)
32
24
16
8
0
Control
AP-PG
Control
AP-PG
D
NC
HFD
p<0.05
n.s.
n.s.
LDL cholesterol (mg/dl)
1200
900
600
300
0
Control
AP-PG
Control
AP-PG
E
NC
HFD
p<0.05
p<0.05
n.s.
oxidized LDL cholesterol (mg/dl)
80
60
40
20
0
Control
AP-PG
Control
AP-PG


A
NC
HFD
p<0.01
p<0.05
p<0.05
MOMA-2 positive area (%)
0
2
4
6
Control
AP-PG
Control
AP-PG
B
NC
HFD
Control
AP-PG
Control
AP-PG