Journal of Geriatric Cardiology (2014) 11: 74–78

Research Article · Open Access ·

Hyperhomocysteinemia independently causes and promotes atherosclerosis in LDL receptor-deficient mice

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Abstract

Background Hyperhomocysteine is an independent risk factor of coronary heart disease (CHD). However, whether hyperhomocysteine affects the progression of atherosclerosis is unclear. In the present study, we examined the effect of hyperhomocysteine on the formation of atherosclerosis in low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice. **Methods** Forty-eight 7-week-old $LDLr^{-/-}$ mice were assigned to the following groups: mice fed a standard rodent diet (control group), mice fed a high-methionine diet (high-methionine group), mice fed a high-fat diet (high-fat group), and mice fed a diet high in both methionine and fat (high-methionine and high-fat group). At the age of 19, 23, and 27 weeks, four mice at each interval in every group were sacrificed. **Results** At the end of the study, mice did not show atherosclerotic lesions in the aortic sinus and aortic surface until 27 weeks old in the control group. However, atherosclerotic lesions developed in the other three groups at 19 weeks. The amount of atherosclerotic lesions on the aortic surface was lower in the high-methionine group than in the high-fat group ($P < 0.001$). Atherosclerotic lesions on the aortic surface in the high-methionine and high-fat group were the most severe. The mean area of atherosclerotic lesions in the aortic sinus compared with atherosclerotic lesions on the aortic surface was lower in the high-methionine group than in the high-fat group ($P < 0.001$). Atherosclerotic lesions in the aortic sinus in the high-methionine and high-fat group were the most severe. **Conclusions** Homocysteinemia accelerates atherosclerotic lesions and induces early atherosclerosis independently in $LDLr^{-/-}$ mice. Reducing the level of homocysteinemia may be beneficial for prevention and treatment of CHD.

J Geriatr Cardiol 2014; 11: 74–78. doi: 10.3969/j.issn.1671-5411.2014.01.013

Keywords: Atherosclerosis; Homocysteine; Low-density lipoprotein; Hyperhomocysteinemia

1 Introduction

Atherosclerosis is a process of chronic pathological changes.[1] High levels of low-density lipoprotein cholesterol (LDL-C) play an important role in the formation of atherosclerosis.[2] Large, long-term epidemiological studies have shown that other factors, such as smoking, family history, diabetes mellitus, and elevated blood pressure, are causes that contribute to the formation of atherosclerosis.[3,4]

In addition to these established risk factors, epidemiological studies have indicated that elevated plasma levels of homocysteine (Hcy) may be an independent risk factor for atherosclerosis and thrombosis.[5,6] Hcy is a non-protein thiol-containing amino acid, which is produced in the cell as an intermediary in methionine metabolism.[7] Hcy is metabolized to cystathione by cystathionine β-synthase (CBS) via the transsulfuration pathway, requiring vitamin B_6. Alternatively, Hcy is remethylated to methionine *via* methionine synthase, requiring vitamin B_{12} as a cofactor and 5′-methyltetrahydrofolate. Consequently, deficiency in enzymes and vitamin B cofactors involved in Hcy metabolism can result in homocysteinemia.

Diet-induced homocysteinemia, by feeding high methionine with low B vitamins[8,9] or Hcy in the drinking water,[10,11] has been shown to accelerate atherosclerotic lesion development in $apoE^{-/-}$ mice. Accelerated atherosclerosis has also been reported using $apoE^{-/-}$ $CBS^{-/-}$ double-knockout mice fed a normal chow diet.[12] However, causality is unproven, and whether hyperhomocysteinemia promotes atherosclerosis remains unknown. Specifically, no studies have determined whether hyperhomocysteinemia can independently induce atherosclerosis, and therefore, accelerated atherosclerosis could result in low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice.

Therefore we examined whether hyperhomocysteinemia

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Received: December 16, 2013 **Revised:** March 20, 2014

Accepted: March 22, 2014 **Published online:** March 26, 2014

accelerates atherosclerotic lesion development, and induces atherosclerosis independently in LDLr$^{-/-}$ mice.

2 Methods

2.1 Mice and diets

Forty-eight 6-week-old male homozygous LDLr$^{-/-}$ mice, backcrossed 10 generations into the C57BL/6 background, were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China).

Mice were maintained on a 12-h light-dark cycle in a pathogen-free environment. After one week of acclimatization, at 7 weeks of age, mice were randomized into four groups as follows: mice fed a standard rodent diet (control group), mice fed a high-methionine diet (hi-met group), mice fed a high-fat diet (hi-fat group), and mice fed a diet high in fat and methionine (hi-met/fat group). The standard rodent diet was bought from Shanghai Laboratory Animal Center and the other three types of diet were made by our laboratory based on the standard rodent diet (the high-methionine diet comprised 1% methionine; the high-fat diet comprised 10% butter fat, 1.25% cholesterol, and 0.5% cholic acid; and the high-methionine and high-fat diet comprised 10% butter fat, 1.25% cholesterol, 0.5% cholic acid, and 1% methionine). Diet and water were given without dose limitations. Food intake and animal body weight were monitored weekly throughout the study. This study was approved by the Institutional Animal Care and Use Committee of Shaoxing Hospital of Zhejiang University.

2.2 End of the study

At the age of 19, 23, and 27 weeks, four mice at each interval in every group were sacrificed and exsanguinated by withdrawing blood from the right ventricle into ethylenediamine tetraacetic acid-coated tubes. Thereafter, the mice were perfused with 4% formaldehyde *via* the left ventricle. The aorta was removed at the starting point of the branch of the inferior renal arteries together with the heart and immersed in fixative for 24 h, before staining with Sudan IV solution or hematoxylin and eosin.

Levels of total cholesterol (TC), LDL-C, and triglycerides (TG) were measured by the Aeroset TM fully automatic clinical biochemical analyzer (Abbott, USA). Hcy was measured by competitive enzyme-linked immunoassay on an autoanalyzer (ADVIA Centaur Immunoassay System, Siemens, Germany).

2.3 Quantitative determination of aortic atherosclerotic lesions

The aorta, which was fixed for 24 h, was placed under a stereomicroscope. The thoracic aorta was removed together with the aortic arch from the ascending aorta at a point 2 mm up the branch of the brachiocephalic artery. The aorta was then cleared of adventitial fat, and opened longitudinally for rinsing in 70% alcohol for 30 s. This tissue was stained with Sudan IV solution for 15 min. Thereafter, tissue was degreased in 80% alcohol for 20 min, and washed in running water for 1 h.[13]

Atherosclerotic lesions were stained with a pinkish appearance and were easily identified. To quantify areas of atherosclerotic lesions, Sudan IV-stained specimens were photographed by a digital camera (Nikon D60 color camera) connected to a stereomicroscope and analyzed using Image Pro Plus 6.0. Mean areas of lesions were calculated as the total atherosclerotic lesion area.

2.4 Anatomical examination

The heart, including the ascending aorta, separated from the aortic arch, was paraffin embedded. The half containing the aortic sinus was sectioned serially at 10 μm intervals until the aortic valve leaflets appeared. Thereafter, every other 4 μm section was collected on glass slides. Five sections taken spanning approximately 300 μm of the aortic sinus from the commissures of the aortic leaflets and upward, were stained with hematoxylin and eosin, and evaluated microscopically. The of area of lesions was measured blindly by the same person using computer-assisted image analysis (Olympus BX50 light microscope and Image Pro Plus 6.0). The amount of atherosclerosis in the aortic sinus was expressed as mean plaque size of the five sections.

2.5 Data analysis

Analysis of variance was used for group comparisons. Statistical significance was set at $P < 0.05$. SPSS 15.0 was used for the calculations. Data are expressed as mean ± SD.

3 Results

3.1 Body weight and diet intake

There was a tendency for mice in the control and hi-met groups to eat more food than in the other two groups. We only recorded the amount taken by each group per week, since there were insufficient data to analyze by Statistical Methods. The mean plot of food intake was presented in Figure 1. Initial body weight was similar among the four groups. However, gradually, at the age of 19, 23 and 27 weeks, mice in the hi-met group gained more weight than the mice in the other two groups (Table 1, $P = 0.049$, $P = 0.002$, $P = 0.001$, respectively).

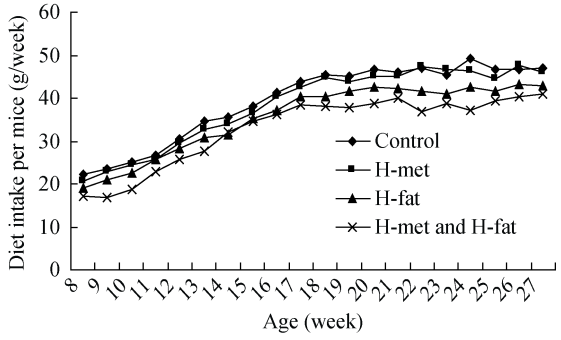

Figure 1. Mean food intake of mice per week. H-met group: mice fed a high-methionine diet; hi-fat group: mice fed a high-fat diet; hi-met/fat group: mice fed a diet high in fat and methionine.

3.2 Homocysteic acid and lipids

Homocysteic acid was higher in the two groups on a diet with methionine supplementation than in the other two groups at the age of 19, 23, and 27 weeks (all $P < 0.001$, Table 1). TC, TG, and LDL-C levels were significantly higher in the groups with a diet high in fat than in the other two groups without fat supplementation (all $P < 0.001$, Table 1).

3.3 Atherosclerosis

At the end of study, the mice did not show atherosclerotic lesions in the aortic sinus and aortic surface until 27 weeks old in the controls. However, atherosclerotic lesions

Table 1. Body weight, lipids, homocysteic acid levels, and the amount of atherosclerotic areas in the aorta and aortic sinus of LDLr$^{-/-}$ mice.

Age (weeks)	Groups				*P*
	Control	H-met	H-fat	Hi-met/fat	
Weights (g)					
Baseline	21.15 ± 1.60	20.80 ± 1.46	20.62 ± 1.78	20.80 ± 2.02	0.897
19	30.28 ± 2.23	30.62 ± 2.92	28.36 ± 2.33	28.63 ± 1.78	0.049
23	32.88 ± 2.77	34.48 ± 2.78	29.80 ± 2.92	30.03 ± 1.68	0.002
27	37.73 ± 1.66	38.68 ± 1.20	33.78 ± 1.49	35.03 ± 1.11	0.001
TC (mg/dL)					
19	7.32 ± 0.63	7.15 ± 0.77	28.79 ± 2.35	27.77 ± 2.53	< 0.001
23	8.37 ± 0.57	8.11 ± 0.78	34.70 ± 1.83	32.89 ± 1.43	< 0.001
27	9.48 ± 0.6	8.92 ± 0.39	43.11 ± 1.30	41.89 ± 2.49	< 0.001
TG (mg/dL)					
19	0.58 ± 0.10	0.61 ± 0.08	1.97 ± 0.14	2.00 ± 0.14	< 0.001
23	0.84 ± 0.09	0.83 ± 0.06	2.55 ± 0.14	2.56 ± 0.12	< 0.001
27	0.87 ± 0.07	0.86 ± 0.08	3.69 ± 0.47	3.91 ± 0.14	< 0.001
LDL-C (mg/dL)					
19	2.74 ± 0.42	2.88 ± 0.21	12.61 ± 0.83	12.09 ± 1.29	< 0.001
23	5.05 ± 0.50	4.43 ± 0.45	21.51 ± 1.45	21.53 ± 1.24	< 0.001
27	6.07 ± 0.45	6.19 ± 0.24	41.03 ± 1.50	40.75 ± 1.81	< 0.001
Homocysteine (μmol/L)					
19	0.83 ± 0.16	7.42 ± 0.50	0.80 ± 0.11	7.85 ± 0.28	< 0.001
23	0.87 ± 0.09	15.85 ± 1.13	0.92 ± 0.10	16.27 ± 0.75	< 0.001
27	1.29 ± 0.22	17.51 ± 0.67	1.23 ± 0.12	17.24 ± 0.73	< 0.001
Aortic lesion areas (mm^2)					
19	0	0.41 ± 0.14	1.00 ± 0.15	1.93 ± 0.10	< 0.001
23	0	0.65 ± 0.20	1.93 ± 0.13	2.50 ± 0.17	< 0.001
27	0.58 ± 0.20	1.02 ± 0.10	2.34 ± 0.13	3.27 ± 0.34	< 0.001
Aortic sinus lesion areas (μm^2)					
19	0	4680.00 ± 480.73	187709.50 ± 17177.69	214522.25 ± 11031.03	< 0.05
23	0	7377.25 ± 593.76	220467.25 ± 13165.64	253470.50 ± 5498.69	< 0.001
27	3351.00 ± 643.53	33468.50 ± 4183.79	235461.75 ± 12537.87	277178.75 ± 10932.76	< 0.001

Data are presented as mean ± SD. Hi-fat: mice fed a high-fat diet; Hi-met: mice fed a high-methionine diet; Hi-met/fat: mice fed a diet high in fat and methionine; LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; TG: triglycerides.

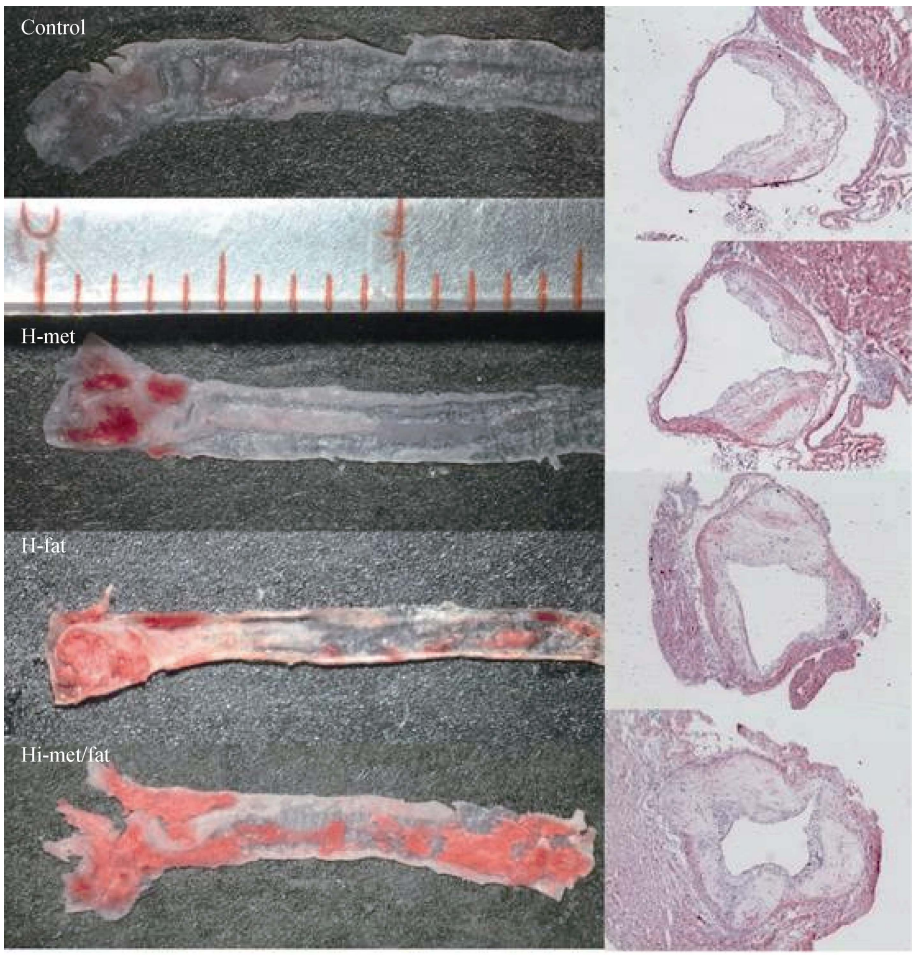

Figure 2. Atherosclerotic lesions in the aorta by en face preparation (A) and of homologous aortic sinus in serial sections (B). The lesions in the aorta were stained with Sudan IV and representative photomicrographs are shown at the age of 27 weeks. Serial sections of the aortic sinus were prepared from each mouse. The tissue sections were stained with hematoxylin and eosin (B = A×50 times in size) and images were taken under a microscope. The lesions in the photographs were traced, and the areas of the lesions were measured. H-met group: mice fed a high-methionine diet; hi-fat group: mice fed a high-fat diet; hi-met/fat group: mice fed a diet high in fat and methionine.

formed by 19 weeks of age in the hi-met group (Figure 2). The hi-fat group and hi-met/fat group also showed atherosclerosis in the aorta and aortic sinus at the age of 19 weeks. However, the hi-met/fat group had more severe atherosclerotic lesions in the aorta and aortic sinus than those in the hi-fat group at the age of 19, 23, and 27 weeks (all $P < 0.001$, Figure 2, Table 1).

4 Discussion

Coronary heart disease (CHD) is the most common cause of death worldwide. CHD is also a major cause of disability, with many people reporting problems, or needing assistance with daily activities.[14] Therefore, determining the reasons for development of atherosclerosis and pursuing methods to treat people with CHD are important goals.

High levels of lipoproteins play an important role in the progression of atherosclerosis.[2] The cure for atherosclerosis is to decrease the levels of lipoprotein by medications, such as statins.[15] Because many other factors participate in the progression of atherosclerosis, such as smoking, and hypertension, elimination of these factors also decrease the occurrence of CHD.[16]

Numerous epidemiological studies have demonstrated that increased concentrations of plasma Hcy are an independent risk factor of atherothrombotic diseases, such as ischemic heart disease, stroke, and peripheral vascular disease.[17] Strong associations between plasma Hcy and atherothrombotic diseases have predominantly been observed in cross-sectional and retrospective case-control studies.[5,6] Some studies have shown that diet-induced homocysteinemia, by feeding high methionine with low B vitamins[8] or Hcy in the drinking water,[10] accelerates atherosclerotic lesion development in apoE$^{-/-}$ mice. In the

present study, we found that homocysteinemia accelerated atherosclerotic lesion development in LDLr$^{-/-}$ mice. Furthermore, hyperhomocysteinemia was found to independently induce atherosclerosis in LDLr$^{-/-}$ mice. Therefore, Hcy may be an independent risk factor, which accelerates atherosclerosis or induces atherosclerosis in LDLr$^{-/-}$ mice.

However, only a weak, or no association, has been found between atherosclerosis and homocysteine in many nested, case-control and prospective studies.[18] These conflicting results suggest that plasma Hcy could be a marker, or a consequence, of atherothrombotic diseases, rather than a cause. In the case of causality, the epidemiological data suggest that Hcy plays a more important role in patients with established disease than in persons without symptoms of vascular disease.[7,19] Therefore, Hcy might promote the complications of atherosclerosis rather than atherosclerosis itself.[19] More studies should be conducted in the future to clarify this issue.

Most researchers believe that Hcy is a risk factor for atherosclerosis. In our previous study, we found that administration of folic acid reduces the level of plasma Hcy in unstable angina patients with hyperhomocysteinemia and improves flow-mediated dilation.[20] However, whether reduction of plasma Hcy reduces mortality rate is unclear.

In conclusion, homocysteinemia accelerates atherosclerotic lesions and induces atherosclerosis independently in LDLr$^{-/-}$ mice. Administering medication that could decrease the level of homocysteic acid in people who are likely to acquire CHD, or have CHD, may be beneficial.

Acknowledgements

This study was supported in part by the Health Ministry Scientific Research Fund of China (No. WKJ2011-2-018) and the Science and Technology Plan Project of Zhejiang Province (No. 2012C33040), China.

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