ИММУНИЗАЦИЯ АПОПРОТЕИНАМИ ЛПВП ИНДУЦИРУЕТ ОПОСРЕДОВАННОЕ Т-ЛИМФОЦИТАМИ ВОСПАЛЕНИЕ АОРТЫ И ВЕН

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Резюме. В последнее время все большее распространение получает гипотеза о том, что атеросклеротические процессы в большей степени обусловлены иммунными (аутоиммунными) механизмами. В то же время аутоиммунная гипотеза атерогенеза не стала общепринятой и требует дополнительных доказательств. Ранее нам удалось вызвать изменения в стенке аорты крысы, подобные изменениям на ранних стадиях атеросклероза человека, а также вызвать висцеральное ожирение у нормохолестеринемических крыс Wistar путем однократной иммунизации нативными липопротеинами высокой или низкой плотности человека. Также нами было обнаружено, что иммунный ответ на нативные ЛПВП человека вызывает атеросклерозоподобные поражения в аорте кролика, такие как адипоцитарная и хондроцитарная метаплазия, отложения протеогликанов, лейкоцитарная инфильтрация. Изменения в стенке аорты кроликов и крыс, иммунизированных нативными липопротеинами, были получены на фоне нормального уровня холестерина крови. Таким образом, иммунный ответ против ЛПВП или ЛПНП может быть независимой причиной атерогенеза. Цель данного исследования состояла в том, чтобы проверить, будет ли иммунизация человеческими апопротеинами ЛПВП (белками апоА1 и апоЕ) вызывать атеросклерозоподобные поражения в аорте нормохолестеринемических крыс Wistar. Апопротеины ЛПВП выделяли из плазмы человека или крысы. Для иммунизации апопротеинами ЛПВП человека использовали крыс Wistar (n = 5) в возрасте 2 месяцев. Апопротеины ЛПВП вводили однократно в виде внутрикожной инъекции по 100 мкг на крысу в неполном адъюванте Фрейнда. Контрольным крысам вводили подкожно неполный адъювант Фрейнда (n = 5). Крыс вскрывали через 25 недель после иммунизации. Срезы аорты окрашивали гематоксилином и эозином для световой микроскопии. Для определения инфильтрации Т-лимфоцитами проводили иммуногистохимическое окрашивание FITC-мечеными антителами, специфичными к крысиному CD3. $CD3^+$Т-лимфоциты выявляли с помощью флуоресцентного микроскопа Olympus BX53. Уровень антител к апопротеинам человека и крысы определяли методом непрямого твердофазного иммуноферментного анализа. Иммунизация апопротеинами ЛПВП вызвала опосредованный Т-лимфоцитами иммунный ответ, без выработки аутоантител к апопротеинам ЛПВП. Интима и адвентиция аорты были инфильтрированы Т-лимфоцитами у крыс, иммунизированных апопротеинами ЛПВП. Неожиданным было обнару-

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жение сильной Т-лимфоцитарной инфильтрации всех слоев стенки вен у крыс, иммунизированных апопротеинами ЛПВП человека. Таким образом, посредством иммунизации апопротеинами нам не удалось вызвать у крыс изменения в стенке аорты характерные для продвинутых стадий атеросклероза. Однако иммунизация апопротеинами ЛПВП вызвала опосредованное Т-лимфоцитами сильное воспаление аорты и вен.

Ключевые слова: воспаление сосудов, апопротеины ЛПВП, аутоиммунное воспаление, Т-лимфоцитарная инфильтрация, экспериментальная модель

HDL APOPROTEIN IMMUNIZATION INDUCES T CELL-MEDIATED VENULITIS AND INFLAMMATION IN AORTA

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Abstract. The hypothesis that atherosclerotic processes are mostly caused by immune (autoimmune) mechanisms has recently been gaining attraction. At the same time, the autoimmune hypothesis of atherogenesis has not become generally accepted and requires additional evidence. Previously, we were able to induce changes in the aortic wall similar to those observed in the early stages of human atherosclerosis, and also to produce visceral obesity in normocholesterolaemic Wistar rats by a single immunization with human native high- or low-density lipoproteins. We also found that the immune response to native human HDL causes atherosclerosis-like lesions in the rabbit aorta, such as adipocyte and chondrocyte metaplasia, proteoglycan deposits, and leukocyte infiltration. Atherosclerosis-like lesions developed in the aorta of hnHDL-immunized rabbits against a background of normal blood LDL-cholesterol level. Thus, an immune response against HDL or LDL may be an independent cause of atherogenesis. The aim of this study was to test whether immunization with human HDL apoproteins (apoA1 and apoE proteins) would induce atherosclerosis-like lesions in the aorta of normocholesterolemic Wistar rats. HDL apoproteins were isolated from human or rat plasma. Wistar rats (n = 5) aged 2 months were used for immunization with human HDL apoproteins. HDL apoproteins were administered as a single intradermal injection of 100 μg per rat in incomplete Freund's adjuvant. Control rats were injected subcutaneously with incomplete Freund's adjuvant (n = 5). Rats were dissected 25 weeks after immunization. Rat aorta sections were stained with hematoxylin and eosin for light microscopy. T lymphocytes infiltration was determined by immunohistochemical staining with FITC-labeled antibodies specific to rat CD3. $CD3^+$T lymphocytes were detected using an Olympus BX53 fluorescent microscope. The level of antibodies to human and rat HDL apoproteins was determined by indirect enzyme-linked immunosorbent assay. Immunization with HDL apoproteins induced a T cell mediated immune response without production of autoantibodies to HDL apoproteins. The aortic intima and adventitia were infiltrated with T lymphocytes in rats immunized with HDL apoproteins. Pronounced T lymphocytic infiltration was found in all layers of the vein wall in rats immunized with human HDL apoproteins. Thus, immunization with HDL apoproteins causes T cell mediated inflammation of the aorta and venulitis.

Keywords: vascular inflammation, HDL apoproteins, autoimmune inflammation, T lymphocytic infiltration, experimental model

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Introduction

A high level of total cholesterol or low-density lipoprotein cholesterol is considered the main cause of atherosclerosis and cardiovascular disease [11]. However, the hypothesis that atherosclerotic processes are mostly caused by immune (autoimmune) mechanisms has recently been gaining attraction [6]. Over the lengthy period during which the autoimmune hypothesis of atherosclerosis has been developed and strengthened, the role of the immune response against oxidized LDL, the heat shock proteins (hsps) of microorganisms, apolipoprotein A-1 (main protein constituent of high-density lipoprotein [HDL]) and vessel wall antigens have been studied [8]. Measurement of circulating autoantibodies directed against native and malondialdehyde (MDA)-modified epitope p210 of apoB-100 (IgG-p210nat and IgM-p210MDA) in relation to early atherosclerosis in a large, European longitudinal cohort study of healthy high-risk individuals provides evidence of involvement of autoantibodies against native and MDA-modified apoB-100 peptide 210 in cardiovascular disease

in humans [7]. Previously, we were able to induce changes in the aortic wall similar to those observed in the early stages of human atherosclerosis, and also to produce visceral obesity in normocholesterolaemic Wistar rats by a single immunization with human native HDL (hnHDL) or hnLDL. Rats immunized with hnHDL or hnLDL that as a result produce antibodies against native lipoproteins were found to have pericardial fat, increased visceral adipose tissue volume, inflammation in the aortic wall as identified by the accumulation of leukocytes therein, and destruction of the intima and disruption of the media structure [4]. Immune response to hnHDL was found to cause atherosclerosis-like lesions in the rabbit aorta such as adipocytic and chondrocytic metaplasia, proteoglycan deposits, leukocytic infiltration. Atherosclerosis-like lesions developed in the aorta of hnHDL-immunized rabbits against a background of normal blood LDL-cholesterol level. Thus, immune response against HDL or LDL may be an independent cause of atherogenesis, and HDL is the potential target of the immune attack that leads to atherosclerosis [5].

Epitopes of apoproteins are considered to be the atherogenic component of lipoproteins. Antibodies against ApoA1 demonstrated positive correlations with atherogenesis [8] and were identified as biomarker with a high potential to predict increased cardiovascular disease risk [13]. High level of anti-Apo A-1 autoantibodies in patients with acute coronary syndrome was revealed [14]. Anti-ApoA-1 IgG might be associated with increased atherosclerotic plaque vulnerability in humans and mice. Passive immunization of atherosclerosis-prone apoE-/- mice with anti-apoA-1 IgG increased both atherosclerotic lesion size [9]. At the same time, the autoimmune hypothesis of atherogenesis has not become generally accepted and requires more evidence.

The aim of this study was to test whether immunization with HDL apoproteins would induce atherosclerosis-like lesions in aorta of normocholesterolaemic Wistar rats.

Materials and methods

Rats and Ethics statement

Female Wistar rats were obtained from the Rappolovo breeding facility (Rappolovo, Russia). Animal experiments were performed in accordance with the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU for animal experiments. The protocol and procedures employed were ethically reviewed and approved by the Bioethics Committee of Udmurt State University (Date 25/04/2022/No. 2202).

HDL apoproteins isolation from human or rat plasma

Healthy human sera were obtained at the Republic Blood Transfusion Station (Izhevsk, Russia). Precipitation HDL apoproteins were performed as previously published [1]. Briefly, to serum was added 10% dextran sulfate to final concentrations 0.05% and 1 M MnCl2 to final concentrations 0.05 M, mixing for 10 minutes. With these concentrations, the LDL and VLDL are completely and selectively precipitated. The precipitate is then removed by centrifugation for 10 min at 6000 g. to the LDL- and VLDL-free supernatant are added 10% dextran sulfate to final concentrations 0.65% and 1 M MnCl2 to final concentrations 0.2 M. Precipitation beings immediately and is complete after 2 hr. The mixture is centrifuged at 15 000 g for 30 minutes. The supernatant is removed and the precipitate is washed in Tris-HCl buffer containing 0.1% dextran sulfate and 0.1 M $MnCl_2$, pH = 7.6. The washed precipitate which contains the HDL is dissolved by stirring the suspension by added Tris-HCl buffer containing 0.2% sodium citrate, 1% NaCl, 0.05 M EDTA and 0.05% Twin-20, pH = 8.2. The mixture is centrifuged at 10 000 g for 5 minutes to remove the white precipitate of manganese oxide.

Delipidation HDL-enriched fraction were performed as previously published [2]. Briefly, an equal volume butanol-diethyl ether (40:60, V/V) was added to HDL-enriched fraction and intensively stirred for 30 minutes. Organic phase was removed by centrifugation at 10 000 g for 10 minutes. Apoproteins containing fraction was applied to Superdex 200 10/300 column equilibrating with Tris-HCl buffer containing 0.05% Twin-20, pH = 8.2. Three fractions were obtaining, first – IgG, second – serum albumin, third – apoproteins. However, third fraction besides apoproteins contains IgG and albumin up to 20% of total protein. Purification apoproteins was performed on Albumin&IgG Depletion column. The purity of the isolated HDL apoproteins was tested by SDS-PAGE. The purity of the preparation was 95.2±3.7%. Isolation and purification of HDL apoproteins was performed on a chromatograph AKTA pure 25 M, provided by the Center for the Collective Use of Scientific Equipment, Udmurt State University.

Immunization

At 2 months of age, Wistar rats (n = 5) were immunized with human HDL (hHDL) apoproteins. hHDL apoproteins were administered as a single intradermal injection of 100 μg per rat in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich). Control rats received a subcutaneous injection of IFA (n = 5).

ELISA of antibodies to rat or human HDL apoproteins (apo-HDL)

Plates (Corning-Costar, Acton, MA, USA) were coated overnight at 4 °C with rat or human apo-HDL (20 μg/ml) in 0.15 mol/L PBS. Plates were blocked with 150 μL of 0.15 mol/L PBS/0.05% BSA/Tween-20. Serum samples were added in serial dilution with PBS/Tween-20 and incubated for 1 h at RT. The plates were then incubated for 1 h at RT with 100 μL of goat anti-rat Ig (IgG, IgM, IgA) conjugated to horseradish peroxidase (IMTEC, Russia). Then the substrate

mixture (5 mL citrate buffer solution [pH 5.0]/3 mg ortho-phenylenediamine/15 mL 3% H_2O_2) was added. Absorbances were read after 15 min at 492 nm.

Tissue preparation and histology

Rats were dissected 25 weeks after immunization, Rat aortic specimens were fixed for 24 hours in immunofix and embedded in paraffin for light microscopy. Cross-sections, 5-μm thick, were stained with hematoxylin and eosin.

Immunohistochemistry

The glass-mounted aorta sections were dewaxed and rehydrated by the standard procedure. For the antigen unmasking sections were autoclaved at 120 °C in a Tris-HCL buffer/0.01% sodium borohydride, pH 7.6, for 20 minutes. After autoclaving, the sections were cooled and treated for 5 minutes with a solution of Sudan black B to prevent autofluorescence caused by lipofuscin. The sections were washed three times in the PBS/Twin-20. Further, the sections were treated with a blocking solution containing 5% milk powder in PBS with Twin-20. Then the sections were incubated with FITC labeled antibodies specific to rat CD3 (Mybiosource, USA) overnight at +4 °C, washed three times in the PBS/Twin-20. $CD3^+$T lymphocytes were detected using an Olympus BX53 fluorescence microscope (Japan) provided by the Center for the Collective Use of Scientific Equipment, Udmurt Federal Research Center of the Ural Branch of the Russian Academy of Sciences.

Results and discussion

Human HDL apoproteins used for rat immunization consisted of apoA1 and apoE proteins. Histological analysis of the aorta of Wistar rats immunized with hHDL apoproteins revealed leukocyte infiltration of the aortic wall (Figure 1A) and veins (Figure 1B). In rats injected with IFA, no leukocytes were found in the aortic wall (Figure 1C) or other vessels.

Immunohistochemical analysis showed that the intima and adventitia of the aorta were infiltrated with T lymphocytes in rats immunized with hHDL apoproteins (Figure 2A, B). Severe T lymphocytic infiltration were found in the all layers of the vein wall in hHDL apoprotein immunized rats (Figure 2C).

In the hHDL apoprotein immunized rats the production of antibodies to the immunogen was

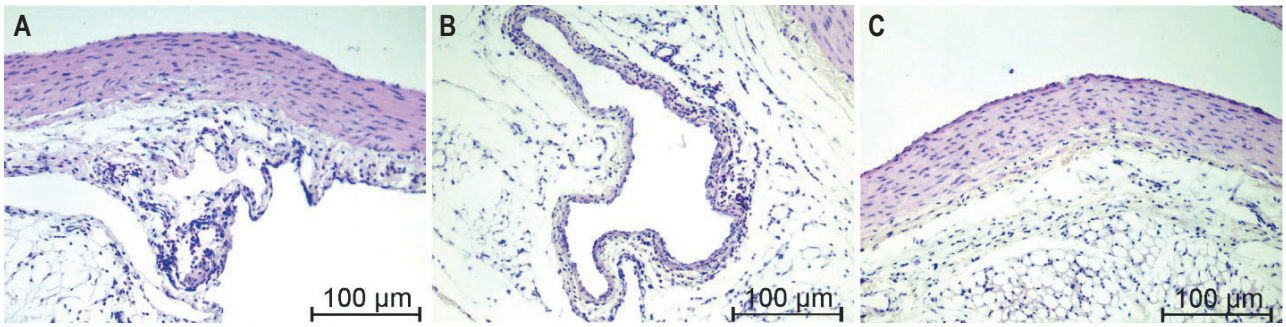

Figure 1. Representative histological specimens of aorta and periaortic tissue of Wistar rats

Note. H&E. A, aorta of rat immunized with hHDL apoproteins. B, leukocyte infiltration of vein of rat immunized with hHDL apoproteins. C, aorta of rat injected with IFA.

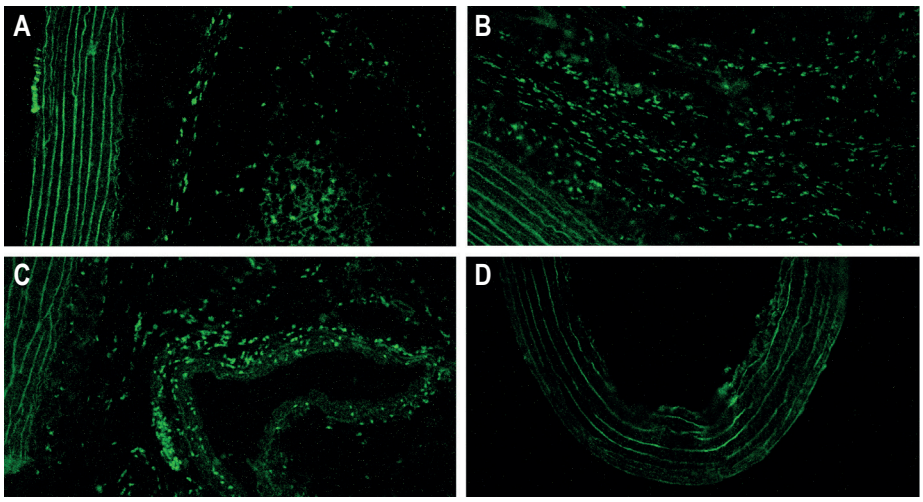

Figure 2. Representative histological specimens of aorta and periaortic tissue stained with FITC anti-rat CD3 antibodies

Note. T lymphocyte infiltrates were found in the intima (A), adventitia (B) of the aorta and in the all layers of the vein wall (C) of rats immunized with hHDL apoproteins. T lymphocytes are absent in the aorta (D) of rats injected with IFA.

observed (Figure 3). Their level continued to remain high 25 weeks after immunization. Autoantibodies to HDL apoproteins were not detected in rats immunized with hHDL apoproteins (Figure 3).

Thus, immunization with human HDL apoproteins resulted in T lymphocytic infiltration of the aortic wall and veins of Wistar rats.

Conclusion

Lymphocytic infiltration of the aorta wall and vein wall is a sign of their inflammation. Since lymphocytic infiltration was detected 25 weeks after immunization, it can be assumed that autoimmune inflammation has become chronic. As is known, T lymphocytic infiltration is the first stage of autoimmune tissue damage in T cell mediated autoimmune diseases [12, 15]. Autoreactive T lymphocytes cause tissue inflammation, damage, or complete tissue destruction [3, 10]. Venulitis in hHDL apoprotein immunized rats was unexpected. Thus, human HDL apoprotein Immunization induces T cell-mediated inflammation in aorta and venulitis.

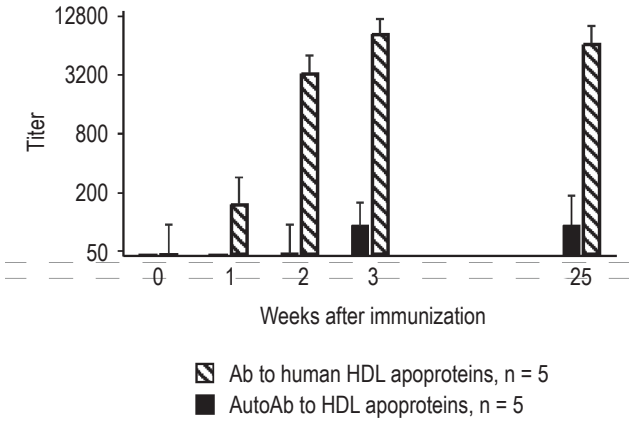

Figure 3. Antibodies to human HDL apoproteins and autoantibodies to HDL apoproteins in Wistar rats immunized with hHDL apoproteins

Note. Data are presented as mean ± SD.

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