Original Article

A New Murine Model for Atherosclerosis with Inflammation in the Periodontal Tissue Induced by Immunization with Heat Shock Protein 60

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It has recently become apparent that the anti-heat shock protein (HSP) antibody plays an important role in the pathogenesis of atherosclerosis. We studied whether immunization with human HSP60 could lead to atherosclerosis in mice. We attempted to induce atherosclerosis in C57BL/6NJcl mice by immunization with human HSP60 under a high-cholesterol diet. The size of fatty streak lesions was significantly enhanced in mice immunized with human HSP60 under a high-cholesterol diet relative to the number in control mice receiving a high-cholesterol diet alone. In addition, these new atherosclerotic model mice showed lesions of inflammation in the periodontal tissue. This new model may thus provide theoretical support for the clinical observation that patients suffering from periodontitis are frequently found to have atherosclerosis. The cytokine ratio of interferon-γ/interleukin-4 in the high-cholesterol diet group was significantly higher than that in the standard chow group (p<0.05). This suggests the presence of a predominantly Th1-type immune response in atherosclerosis. (Hypertens Res 2000; 23: 475-481)

Key Words: atherosclerosis, heat shock protein, periodontal disease, Th1/Th2

Introduction

In an up-to-date review of atherosclerosis, Ross affirmed that atherosclerosis is an inflammatory disease (1). Several investigations have also shown that the immune system is involved in atherosclerosis (2), and that T cells are present in the atherosclerotic lesions of fatty streaks (3, 4). Recently, there has been some investigation of a possible association between heat shock protein 65 (HSP65) and atherosclerosis. Immunization of normocholesterolemic rabbits with HSP65 leads to the formation of atherosclerotic lesions (5). We therefore tried to develop a new model of atherosclerosis in mice by immunization with human HSP60 under a high-cholesterol diet (HCD).

Materials and Methods

Mice

Female C57BL/6NJcl mice and female BALB/c mice, 8 weeks old, were obtained from Clea Japan, Inc. (Tokyo, Japan). This strain was chosen because female C57BL/6 mice were the most susceptible to atherosclerosis among the three strains (C57BL/6, BALB/c, and C3H) studied by Paigen et al. (6).
Diet
The standard chow (NMF; Oriental Company, Ltd., Tokyo, Japan) contained 4% fat by weight (0.1% cholesterol). The high-cholesterol diet, prepared according to the method described by Nishina et al. (7), was composed of 15% cocoa butter, 50% sucrose, 20% casein, 1% corn oil, 5.07% cellulose, 5% AIN-76 mineral mix, 1% AIN-76 vitamin mix, 1% choline chloride, 0.3% dl-methionine, 0.13% dl-α-tocopherol, 0.5% sodium cholate, and 1% cholesterol.

Antigen
We prepared recombinant heat shock protein 60 (HSP60) as an antigen. This recombinant HSP60 was obtained from StressGen Biotechnologies Corp. (Victoria, Canada).

Adjuvant
Complete Freund’s adjuvant (CFA) and Incomplete Freund’s adjuvant (IFA) were obtained from DIFCO Laboratories (Detroit, MI).

Immunization
Mice were immunized subcutaneously 3 times (once every 3 weeks) as follows. In the 1st week, they were immunized with human HSP60 (1 μg/mice/dose) and CFA. In the 4th and 7th week, they were immunized with human HSP60 (1 μg/mice/dose) and IFA.

Streptozotocin-Induced Diabetes
The mice received a single intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) at dose of 180 mg/kg in citrate buffer, pH 4.5. Whole blood samples were collected from the tail veins at 10 AM on the fifth day after streptozotocin treatment. During bleeding, the blood glucose levels were immediately determined by the glucose dehydrogenase method on a portable glucometer (Advantage; Yamanouchi, Tokyo, Japan). Mice showing blood glucose levels of 200 mg/dl or more were considered diabetic and used in this experiment.

Quantitative Assessment of Atherosclerotic Lesions
For quantitative assessment of atherosclerotic lesions, we used a modification of the method of Paigen et al. (8). In brief, the heart and upper portion of the aorta were removed from the animals and the peripheral fat was carefully separated. After fixation in 10% buffered formalin, the upper section of the heart was embedded in OTC compound and frozen. Frozen sections were discarded until the 3 valve cusps of the aorta appeared. The presence of the 3 valve cusps of the aorta was determined by examining sections microscopically with toluidine blue stain. Once the appropriate section was located, sectioning continued along the ascending aorta away from the heart until the valve cusps were no longer visible. The 4-μm sections were fixed on gel-coated microscope slides, stained with Oil red O, and counterstained with hematoxylin. Lesion areas per section were counted on the grid (UOCMSQ10/10; OLYMPUS, Tokyo, Japan). Mean aortic lesion size was defined as the average area stained with Oil red O.

Detection of Anti-HSP60 Antibody
Solid-phase enzyme-linked immunosorbent assays (ELISA) were used to measure titers of anti-HSP-60 antibody. The 96-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were coated with 4 μg/ml HSP-60 in 20 mmol/l bicarbonate buffer (pH 9.5) overnight at 4°C. Wells were blocked with 0.5% bovine serum albumin (Sigma) at room temperature for 2 h, then diluted sera were applied and the mixtures were incubated at room temperature for 2 h. Bound antibodies were detected with appropriate horseradish peroxidase-conjugated anti-mouse immunoglobulin (IgG2a; Pharmingen, San Diego, CA). All reactions were developed with 0.3 mg/ml 2,2’-azino-bis-(3-ethylbenzthiozolin-6-sulfonic acid) (Sigma) in 0.1 M citrate buffer (pH 4.35) containing 0.03% H2O2 for 30 min and were read at optical density (450 nm) with a plate reader (BioRad, Hercules, CA).

Cytokines Analysis
Cytokines were determined with commercial ELISA kits Cytoscreen®, Biosource International, Camarillo, CA. These commercial ELISA kits of interferon-γ (IFN-γ) and interleukin-4 (IL-4) were purchased from Biosource International. At sixteen weeks of treatment, the remaining mice were euthanized (16th week group). Spleen cell suspensions were cultured at 4 × 10⁶ cell/ml (1 ml/well) in 24-well plates at 37°C for 36 h in RPMI 1640 Medium containing 10% fetal calf serum and anti-CD3 antibody (1 μg/ml). Culture supernatants were harvested for measurement of IFN-γ and IL-4. Culture supernatant, IFN-γ and IL-4 cytokine standards (100 μl) were serially diluted and added to the plates. The plates were incubated for 2 h at 37°C and then washed four times. Biotin conjugate (100 μl) was added and incubated for 30 min at 37°C and then washed four times. Streptavidin-HRP working solution (100 μl) was added and incubated for 30 min at room temperature, and then the mixture was again washed four times. Stabilized chromogen (100 μl) was added and incubated for 30 min at room temperature. Finally, stop solution (100 μl) was added and the plates were read at opti-
Measurement of Cholesterol

Serum was obtained from the group of mice euthanized 16 weeks after the beginning of the experiment. The total serum cholesterol levels were determined with a cholesterol measurement kit (Cholesterol E-test; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical Analysis

All values were expressed as mean ± SEM. Statistical analysis was performed using the Student’s t-test and analysis of variance (ANOVA). When appropriate, Tukey’s test was used for multiple comparisons. A value of p < 0.05 was accepted as statistically significant.

Results

Study I: Normal Diet and Anti-HSP Antibodies

First, we attempted to induce atherosclerosis in C57BL/6NJcl and BALB/c mice by immunization with human HSP60 under a standard diet.

In the first experimental design, mice were fed with a standard chow. Twenty-five female C57BL/6NJcl mice were divided into two groups. One group suffered from streptozotocin-induced diabetes mellitus. The other group did not have diabetes mellitus. Mice were immunized with human HSP60, and given 0.45% NaCl in water instead of normal water. Twenty-six female BALB/c mice were divided into two groups. One group suffered from streptozotocin-induced diabetes mellitus. The other group did not have diabetes mellitus. Mice were immunized with human HSP60, and given 0.45% NaCl in water instead of normal water. In the 9th week, both BALB/c and C57BL/6NJcl mice were euthanized. There were no atherosclerotic fatty streaks in either BALB/c or C57BL/6NJcl mice. However, levels of anti-HSP antibodies were higher in the mice immunized with HSP60 than in control mice (data not shown).

Study II: High-Cholesterol Diet and Anti-HSP Antibodies

Experimental Design

Forty-six female C57BL/6NJcl mice were divided into two groups. Mice in the 10th and 16th week groups were euthanized 10 and 16 weeks after the beginning of the experiment, respectively. The 23 mice of each of these groups were further divided into four subgroups as follows. Five mice were fed a standard chow (intact group), 6 mice were fed a high-cholesterol diet (control group), 6 mice were fed a high-cholesterol diet and were immunized with human HSP60 (CH group), and 6 mice were fed a high-cholesterol diet, immunized with human HSP60, and given 0.45% NaCl in water rather than standard drinking water (NCH group).
Quantification of Atherosclerotic Lesions
The mice in the 10th week group were euthanized 10 weeks after the beginning of the experiment. Qualitative analysis of cross-sections of the aortic roots of the mice showed atherosclerotic fatty streak lesions in the control group, the CH group and the NCH group. There were no atherosclerotic fatty streaks in the intact group. Mean aortic lesion sizes for the 16th week group were as follows: intact group, 0.5±1.118 μm²; control group (high-cholesterol diet), 189.4±59.38 μm²; CH group (high-cholesterol diet and immunization with human HSP60), 297.0±125.0 μm²; and NCH group (high-cholesterol diet, immunization by human HSP60, and supplementation of drinking water with 0.45% NaCl), 386.0±106.8 μm². Significantly enhanced fatty streak lesions, compared with the control group, were evident in the CH group (p<0.05) and in the NCH group (p<0.002) (Figs. 1 and 2).

Observation of Periodontal Tissue
We next examined changes in inflammation of the periodontal tissue. In the intact group and the control group, there was no inflammation in the periodontal tissue. In the CH group, inflammation in the periodontal tissue was seen in 4 out of 6 mice. In the NCH group, inflammation in the periodontal tissue was also seen in 4 out of 6 mice. Inflammatory changes in the CH group and the NCH group were mainly suppurative, consisting of neutrophilic infiltration, but chronic inflammation was also seen in a few mice (Fig. 3A, B). Furthermore, in the CH group, foamy histiocytes were also found in the periodontium (Fig. 3C).

There were no significant pathological findings in the other organs (liver, kidney, pancreas, brain, lung, heart, aorta, tooth, or skin).

Quantification of Anti-HSP60 Antibody
The levels of anti-HSP60 antibody in the CH group and in the NCH group were significantly higher than in the control group. The level of anti-HSP60 antibody was 0.009±0.004 (O.D. 405 nm) in the intact group, 0.027±0.019 in the control group, 0.877±0.496 in the CH group, and 0.753±0.418 in the NCH group. The levels of anti-

Fig. 2. Quantification of atherosclerotic lesions in the 16th week group. Significantly enhanced fatty streak lesions, when compared with the control group, were evident in the CH group (p<0.05) and in the NCH group (p<0.002). Data are presented as mean±SEM.

Fig. 3. Cross-section of the periodontal tissue of a mouse in the 16th week group immunized with human HSP60 under a high-cholesterol diet (CH group). A) HE stain, ×100, B) HE stain, ×100. Perivascular infiltrations of lymphocytes were found in the periodontal tissue. C) HE stain, ×50. Neutrophils and lymphocytes were infiltrated in the periodontium intermingled with foamy histiocytes.
HSP60 antibody were high in mice immunized with human HSP60. These results indicate that levels of anti-HSP60 antibody might be increased in mice immunized with human HSP60 (Fig. 4).

Quantification of Cytokines
The level of IFN-γ production of splenocytes was 26.1 ± 8.764 pg/ml in the intact group, 217.2 ± 69.12 pg/ml in the control group, 88.37 ± 16.01 pg/ml in the CH group, and 107.9 ± 37.56 pg/ml in the NCH group. The levels of IFN-γ in the control group (p<0.05) and the CH group (p<0.05) were significantly higher than that in the intact group. The level of IFN-γ in the CH group was significantly lower than that in the control group (p<0.05) (Fig. 5A).

The level of IL-4 in the CH group (199.3 ± 48.61 pg/ml) was significantly higher than that in the intact group (78.1 ± 13.94 pg/ml) (p<0.05). The level of IL-4 in the CH group was significantly higher than that in the NCH group (113.4 ± 11.00 pg/ml) (p<0.05) (Fig. 5B).

The ratio of IFN-γ/IL-4 was 2.023 ± 0.476 in the control group, and 0.336 ± 0.082 in the intact group. The ratio of IFN-γ/IL-4 in the control group was significantly higher than that in the intact group (p<0.05). This demonstrated the presence of a predominantly Th1-type response in atherosclerosis (Fig. 5C).

Quantification of Serum Cholesterol
The level of serum cholesterol were 171.3 ± 5.32 mg/dl in the control group, 176.3 ± 8.58 mg/dl in the CH group, and 173.2 ± 5.50 in the NCH group. The levels of serum cholesterol in the control group (p<0.0001) and the NCH group (p<0.0001) were significantly higher than that in the intact group.

Discussion
In this study, we established a new model of atherosclerosis in mice immunized with HSP60. Systemic pathological analysis of the mice organs revealed inflammation of the periodontal tissue.

There were no atherosclerotic fatty streaks in the mice with streptozotocin-induced diabetes who received a standard diet. In a study by Kunjathoor et al., the size of atherosclerotic lesions did not differ between C57BL/6 mice treated with streptozotocin and those treated with citrate, suggesting that diabetes does not contribute to atherosclerosis in this line (9).

Numerous studies have suggested that salt intake may play an important role in the development and maintenance of hypertension (10, 11). Because hypertension is a major risk factor of atherosclerosis, we also performed a salt-loading trial, with the result that the size of atherosclerotic lesions was increased in the salt-loading group (NCH group). This finding suggests that salt-loading may be enhanced by hypertension, thereby causing the size of atherosclerotic lesions to be accelerated.

There have recently been remarkable findings about the association between heat shock protein 60 (HSP60) and
atherosclerosis (12). The HSPs are a family of peptides characterized by a sequence which is highly conserved along the evolutionary scale. The function of HSPs is to act as molecular chaperones. These peptides are induced by various stressors such as infection, high temperature, free radicals or mechanical stress, and protect cells from environmental insult. In addition, autoimmune responses are evoked by cross-reaction between the HSPs of microorganisms and cellular self components (13). In 1992, immunization of normocholesterolemic rabbits with HSP65 was shown to lead to the formation of atherosclerotic lesions (5). In 1993, Xu et al. showed that there was a strong correlation between anti-HSP65 antibodies and human carotid atherosclerosis (14). In 1995, Schett et al. showed that autoantibodies against HSP60 mediated endothelial cytotoxicity (15). The latter authors concluded that human serum anti-HSP65 antibodies reacted with HSP60 on stressed endothelial cells and were able to mediate endothelial cytotoxicity. Xu et al. concluded that HSP might be one of the antigens inducing atherosclerosis in rabbits (5), and George et al. showed that HSP might be one of the antigens inducing atherosclerosis in mice (16). In the present study, we demonstrated that use of a high-cholesterol diet was required for induction of atherosclerosis by human HSP60 in mice.

Previous studies have suggested an association between periodontal disease and atherosclerosis (17, 18). In 1989, Mattila et al. performed a case-control study in which they found a strong association between dental disease — including periodontitis and dental caries — and acute myocardial infarction (19). In 1989, Syrjanen et al. suggested an association between dental disease — including periodontitis and dental caries — and acute myocardial infarction (19). In 1989, Syrjanen et al. suggested an association between dental infection and ischemic cerebrovascular disease in patients under 50 years of age (20). Atherosclerosis is the principal underlying cause of acute myocardial infarction and ischemic cerebrovascular disease. Thus dental infection and atherosclerosis are closely linked. In 1997, Schett et al. reported that the levels of specific salivary IgA antibodies against mycobacterial HSP 65 were significantly increased in patients with gingivitis when compared to clinically healthy subjects (21).

This data suggested an association between HSP 65 and periodontal disease. Our data also suggested such an association. Our present data on mice treated with HSP showed that the changes in inflammation of periodontal tissue were mainly suppurative. Salivary antibodies against HSP may react with bacterial HSP, causing production of immune complexes of HSP and anti-HSP antibodies in the periodontal tissue. The periodontal tissue may be damaged by Arthus-type immune reactions, and then further complicated by bacterial infection.

Helper T cells can be classified as Th1 or Th2 cells by their cytokine secretory pattern. The Th1 T cell secretes cytokines IFN-γ, tumor necrosis factor-α (TNF-α) and IL-2. In contrast, the Th2 cell secretes cytokines IL-4, IL-3, IL-10 and IL-13. In 1997, Frostegard et al. demonstrated the presence of a predominantly Th1-type T cell response in atherosclerosis (22). Our current data also demonstrated that the ratio of IFN-γ/IL-4 in the control group was significantly higher than in the intact group (p <0.05). This suggested evidence of a predominantly Th1-type T cell response in atherosclerosis.

Recently, there have been several reports on the relationship between atherosclerosis and infection by Chlamydia pneumoniae (23-25), and their results indicate that C. pneumoniae may contribute to atherosclerosis. In 1999, Hu et al. found that infection with the C. pneumoniae AR39 strain in the presence of a high-cholesterol diet significantly exacerbated hypercholesterolemia-induced atherosclerosis in mice (26). Kol et al. reported that both chlamydial and human heat shock protein 60 activated human vascular endothelium, smooth muscle cells, and macrophages (27). Infection with C. pneumoniae, which triggers formation of autoantibodies against HSP60 and thereby results in inflammation, may play an important role in initiation of endothelial injury. The dysfunction of the endothelium permits monocyte infiltration and deposition of lipid-laden macrophages into the subendothelial layers, which is one of the important steps of atherogenesis.

Although George et al. recently found that fatty streak formation in C57BL/6J mice was enhanced by immunization with recombinant HSP65 and HSP65-rich Mycobacterium tuberculosis (16), they did not investigate inflammation in the periodontal tissue. In the present study, we experimentally provided the first theoretical explanation for the clinical observation that patients suffering from periodontitis frequently have atherosclerosis. In the future, we will use this mouse model to study the prevention and regression of atherosclerosis.

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