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ORIGINAL PAPER

Evidence of a role for both anti-Hsp70 antibody and endothelial surface membrane Hsp70 in atherosclerosis

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Abstract Although previous studies have shown that autoantigens such as Hsps have been implicated by induction of an autoimmune process in the development of atherosclerosis, the exact role of anti-Hsp70 antibody in atherosclerosis is unknown. In the present study, the levels of anti-Hsp70 autoantibodies and oxidized low density lipoprotein (OxLDL) were all significantly increased, and they were strongly correlated in an atherosclerosis model. After the endothelial cells were incubated with 20 μg/mL OxLDL for 12 h at 37 °C and followed by 90 min recovery, Hsp70 positive staining of OxLDL-treated endothelial cells was observed on the cell surface in immunostaining and flow cytometric analysis. This membrane Hsp70 was not from culture supernatant Hsp70 and binding of extracellular Hsp70 but was defined as endothelial surface membrane Hsp70. Furthermore, only in the OxLDL-treated group, but not in the untreated group, ^{51}Cr-labeled endothelial cells were lysed by anti-Hsp70 antibody (BD091, Ig^{AS}) in the presence of either complement or peripheral blood mononuclear cells. Control antibodies, including Ig^{Nor}, mAb to Hsp70 (SPA-810), and mAbs to Factor VIII, α-actin, and CD3 showed no cytotoxic effects. In conclusion, anti-Hsp70 antibodies could be reacting with the endothelial surface membrane Hsp70 induced by OxLDL and were able to mediate endothelial cytotoxicity. There is a possibility that a humoral immune reaction to endothelial surface membrane Hsp70 may play an important role in the pathogenesis of atherosclerosis.

Keywords Endothelial cell · Surface membrane Hsp70 · Anti-Hsp70 antibody · OxLDL · Atherosclerosis

Abbreviations

Hsps	Heat shock proteins
OxLDL	Oxidized low density lipoprotein
AS	Atherosclerosis
Ig^{AS}	Atherosclerotic rat plasma Hsp70 autoantibodies
Ig^{Nor}	Control rat plasma Hsp70 autoantibodies
mAb	Monoclonal antibody
BD091	Monoclonal antibody to Hsp70 (which recognized Hsp70 C-terminal)
SPA-810	Monoclonal antibody to Hsp70 (binding site, aa437-504)
PBS	Phosphate-buffered saline
HRP	Horseradish peroxidase
TMB	Tetramethyl benzidine
ELISA	Enzyme-linked immunosorbent assay

Introduction

Atherosclerosis (AS) is a main cause of morbidity and mortality, in which inflammation and autoimmunity are associated with pathogenesis of the disease (Wick et al.

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2004). Heat shock proteins (Hsps) are identified as a possible autoantigenic determinant and present as self-antigens to the immune system, resulting in the production of autoantibodies, which may play a significant role in the development of atherosclerosis (Hsps) (Xu 2002). Although work from a number of groups has found that circulating Hsp60/Hsp65 and their autoantibody levels are associated with the severity of atherosclerosis (Xu et al. 1999; Prohaszka et al. 2001; Zhu et al. 2004; Schett et al. 1995), studies have reported that the inverse is true for Hsp70, i.e., that circulating Hsp70 appears to confer protection against cardiovascular disease and vascular cell damage (Zhu et al. 2003). Higher anti-Hsp70 antibody levels were found in patients with intermittent claudication, critical lower limb ischemia, and aneurysms, suggesting that anti-Hsp70 might be involved in the pathogenesis and procession of cardiovascular disease (Chan et al. 1999). In another study, Pockley et al. demonstrated that although increases in intima-media thicknesses (as a measure of early atherosclerosis) at follow-up were less prevalent in subjects having high serum Hsp70 levels (75th percentile) at the time of enrollment, no such relationship was seen for circulating anti-Hsp antibodies (Pockley et al. 2003). Zhu et al. found no association between anti-Hsp70 IgG seropositivity and the prevalence of coronary artery disease (Kocsis et al. 2002; Zhu et al. 2003). Lower anti-Hsp70 antibody levels were found in the atherosclerosis group (Dulin et al. 2010) and in the acute coronary syndrome group (Zhang et al.). Meanwhile, Yuan found that the increase of anti-Hsp70 was associated with the independent risk of increased electrocardiography abnormality, which suggested that anti-Hsp70 might be involved in possibly cardiovascular diseases (Yuan et al. 2005). These studies suggest that the role of anti-Hsp70 antibodies in cardiovascular disease is controversial. The exact role of Hsp70 antibodies in the progression of atherosclerosis is so far unclear.

We have previously shown that anti-Hsp70 antibody (BD091) promotes atherosclerosis in rats (Leng et al. 2010). An important question that remains unanswered is how does BD091 promote atherosclerosis? Although Hsp70 is considered intracellular, cell membrane expression is observed in response to various types of stresses on tumor cells (Singh-Jasuja et al. 2001; Srivastava 2002; Campisi et al. 2003; Enomoto et al. 2006). In the present study, we aimed to identify the evidence of a role for both anti-Hsp70 antibody and endothelial surface membrane Hsp70 in atherosclerosis.

Materials and methods

Animals

All animal experiments were performed according to protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals. SD rats were maintained on a light/dark (12/12 h) cycle at 22 °C and received food and water ad libitum. All the animals used in this work received humane care in compliance with institutional animal care guidelines, and the care was approved by the Local Institutional Committee.

Induction of atherosclerosis and pathological study

Each of two groups consisted of eight rats that were fed either a normal diet (Normal group, Nor group) or a high-cholesterol diet (Atherosclerosis group, AS group) (Cai et al. 2005). The high-cholesterol diet contained 3 % cholesterol, 0.5 % cholic acid, 0.2 % 6-propyl 2-thiouracil, 5 % sucrose, 3 % (w/w) lard, and 81.3 % regular rat chow, with the addition of a single dose of vitamin D3 (600,000 unit/kg, i.p.) (Sigma, China, Beijing). At the end of treatment, rats were anesthetized by intraperitoneal injection of pentobarbital natrium (50 mg/kg body weight; Sigma, China, Beijing). The aorta was fixed and removed for hematoxylin and eosin (HE staining).

Venous blood samples from all rats were obtained. Plasma OxLDL (Northwest Life Science Specialties, Vancouver, WA) (Rapidbio Biosource R&D) was assayed using ELISA kits according to the manufacturer's instructions. Plasma anti-Hsp70 autoantibodies were measured by using kits supplied by Enzo (Enzo Life Sciences, Inc., ADI-EKS-750). Titers of plasma Hsp70 antibody classes were determined by ELISA. In brief, microtiter plates were coated with recombinant Hsp70 (Enzo Life Sciences, Inc., SPP-758D), dissolved in PBS at a concentration of 1 μg/ml overnight at 4 °C, and then washed three times. After blocking with 5 % BSA overnight at 4 °C, plates were washed three times. 100 μl samples in duplicate were added to the plate wells and incubated at 37 °C for 30 min. Wells were washed three times with 1× PBST (PBS/0.5 % Tween-20). Then, 100 μl (1:1,000 in PBS; horseradish peroxidase (HRP)-conjugated) goat anti rat IgM (Bethyl Laboratories, A110-100P), IgA (A110-102A), and IgG (Abcam, UK, ab6120) were added to the plates, which were incubated at 37 °C for 20 min. One hundred microliters of TMB substrate was added to each well and incubated at 37 °C for 15 min. ELISA was developed using 100 μl per well and 2 M H_2SO4 and read at 405 nm with a 492-nm reference filter.

OxLDL

Oxidized low density lipoprotein (OxLDL) was prepared according to the method of Huber et al. (1990). A 10-mg sample of *n*-low density lipoprotein (LDL) (Sigma, United States) was dialyzed against Tris/NaCl Buffer (50 mmol/L Tris in 0.15 mol/L NaCl, pH8.0) to remove the EDTA. Tris/NaCl buffer was added to the dialyzed *n*-LDL to adjust

the protein concentration to 30 mg/mL. A 1-mL aliquot of 20 mmol/L $CuSO_4$ was added to 1 mL of dialyzed *n*-LDL. Oxidation at 37 °C was followed spectrophotometrically (234 nm) over a period of 24 h until oxidation was complete. The OxLDL was then dialyzed at 4 °C with 4 L Tris buffer, filtered with a 0.22 nm filter, and stored under nitrogen at 4 °C. Oxidation was monitored by the use of measurements of TBARS. Briefly, LDL was incubated with thiobarbituric acid (0.5 wt/vol, in H_2SO_4, 50 mM) for 30 min at 100 °C. The solution then was centrifuged for 5 min, and the difference in absorbency at 532 and 580 nm was calculated. TBARS concentration was determined as MDA equivalents with the use of an MDA standard curve.

Endothelial cell culture and reagents

Rat aortic endothelial cells (RAECs) were prepared as described previously (Kreisel et al. 2001). Mainly, adult male rats were sacrificed by cervical dislocation and a thoracotomy was performed under sterile conditions. The full length of the aorta was removed and cut coronally into six circular segments. The segments were washed in PBS to remove blood from the intra-aortic lumen, opened longitudinally with microscissors, and placed endothelial side down onto the collagen gel. Thirty-six hours later, after adequate adherence between aortic endothelium and collagen, 1 ml of complete endothelial cell medium was carefully added to each well so as not to disturb the segments. Within 3–5 days, after visible cellular out-growth from the aortic segments, the medium was aspirated and the aortic segments removed in a sterile fashion. After reaching confluence, passage 1 (P1) cells were released from the T25 flasks by a 10–15-min incubation in 5 mM EDTA, and split at a 1:3 ratio into a T75 flask. Passage 2 (P2) cells were either split at a 1:3 ratio or used for endothelial cell characterization. All experiments were performed with passage 2 (P2) to passage 5 (P5) endothelium. RAECs were characterized by immunochemical staining using a monoclonal antibody (sc-59957, Santa Cruz, California) for endothelial cell-specific VIII factor relative antigen. The endothelial cells were sub-cultured in medium-199 supplemented with 10 % heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 mg/ml) in a humidified CO_2 incubator. RAECs from the third and fifth passages were used for all the experiments.

Purification of plasma Hsp70 autoantibody

Purification of atherosclerotic rat plasma anti-Hsp70 autoantibodies was performed following an established method (Mayr et al. 1999). The Hsp70 coupling was performed using Affi-Gel 15 kit (153-6051; Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. Briefly, 3 mg recombinant Hsp70 (Enzo Life Sciences, catalog number: ADI-ESP-502-F) solubilized in Hepes buffer (0.1 M) were incubated with 2 mL agarose gel beads under constant gentle agitation for 4 h at 4 °C. Thereafter, 100 μl of ethanolamine-HCl (1 M, pH8.0) was added and the gel was incubated for another hour to block protein binding activity. The coupled gel was transferred to the column, washed with Hepes buffer, and equilibrated with PBS, pH7.2.

Pooled atherosclerotic rats plasma were inactivated at 56 °C for 30 min and precipitated with 50 % saturated $(NH_4)_2SO_4$. The precipitated Igs (5 mg/ml), dialyzed against PBS overnight, were added to the column, incubated for 30 min at room temperature, and washed to remove unbound material monitoring with OD 280 nm. When no more proteins were detectable in the effluent, 6 ml of 20 mM HCl was added. The eluant was fractionated in 300 μl portions and immediately neutralized with 0.5 M NaOH. Fractions with an OD value over 0.25 nm were pooled, concentrated, dialyzed against PBS, and filtered. This entire procedure was repeated five times, and 3.1 mg of Ig was thus obtained from 16 ml of the original serum. Anti-Hsp70 reactivity of the purified Ab, measured by the ELISA method described above, was similar to that of the original plasma (1:2,560). The concentrations of purified antibodies were determined using a protein assay from Bio-Rad (Protein Assay Kit I #500-0001, US).

Membrane preparation and western blotting

OxLDLs exhibit a variety of atherogenic properties including cell cytotoxicity at low concentrations and apoptosis at higher concentrations. To confirm the dose–effect relationship between Hsp70 in membrane and cell damage, plasma membranes were prepared using a modification of the procedure of Bauer and Hurtenbach (1986). RAECs cells (90 % confluent, 10-cm culture dish) were harvested in 1 ml of cold TNE buffer (10 mM Tris–HCl (pH7.5), 150 mM NaCl, 5 mM EDTA 4 mg/ml trypsin inhibitor, 1 mg/ml benzamide, 5 μmol/ml leupeptin, 200 μM sodium vanadate, 100 nM okadaic acid, and 1 mg/ml PMSF). The cell pellet was resuspended in ice-cold isotonic 3 mM phosphate-buffered sucrose solution (0.32 M, pH7.6, ImM EDTA). The cells were homogenized in a Dounce homogenizer by 50 strokes with a tight pestle. After centrifugation (2,000×*g*, 4 °C, 15 min) the pellet was again homogenized as described above. Pooled supernatants were spun at 11,000×*g* at 4 °C for 25 min and the resulting supernatant was again centrifuged at 20,000×*g* at 4 °C for 45 min. The pellet, containing plasma membranes and associated proteins, was resuspended in 0.1 ml PBS and stored at −70 °C. The purity of the fractions was tested using an enzyme kit (Sigma, 545-A) that detects gamma-glutamyl-transferase. Purified fractions

were used for further analysis only if the activity of this enzyme was detectable in the membrane fraction but not in the cytoplasmic fraction. Protein concentrations were determined using a protein assay from BioRad. Equal protein amounts (20 μg) were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore Corp., Bedford, MA, USA).

To analyze whether Hsp70 was present within the membrane preparation, the PVDF membranes were blocked for 2 h at room temperature with 3 % gelatin in Tris-buffered saline (500 mmol/L NaCl and 20 mmol/L Tris, pH8.0), and then incubated with mAb against Hsp70 (BD091, 1:80 in Tris-buffered saline containing 1 % gelatin and 0.05 % Tween 20) (Leng et al., 2010), HSC70 (SPA-815, Stressgen) for overnight at 4 °C. The purity of the fractions was tested using HSC70 antibody which detected HSC70 in the cytoplasmic fraction but not in the membrane fraction. After 1 h incubation at room temperature with horseradish peroxidase-conjugated secondary antibody (1:5,000) (Santa Cruz), immunoreactive proteins were visualized by enhanced chemiluminescence reagent (Santa Cruz). Results of representative chemiluminescence were scanned and densitometrically analyzed using ImageMaster VDS system (Amersham, UK) with the help of the Imagequant TL site program.

Immunofluorescence

Immunofluorescence studies were performed as described with slight modifications (Xu et al. 1994). Subconfluent endothelial cells (1×10^6) were grown on four sterile glass cover slide were incubated at 20 μg/mL OxLDL for 12 h at 37 °C and followed by 90 min recovery, and then at 37 °C for 3 h. For surface staining, cells were washed twice with PBS and incubated with 100 μl of primary antibody (BD091), Isotype-control antibody (IgG1, #5415, Cell Signaling Technology) for 1 h at room temperature. After three washes with PBS, the cells were incubated with a rabbit anti-mouse Ig-FITC conjugate (catalog number: 81-6711, Invitrogen) for 30 min, fixed with absolute methanol for 5 min, rinsed, and embedded in *n*-propylgalat/glycerol (P3130; Sigma). The slides were examined using a fluorescent microscope.

Flow cytometric analysis

Quantificative flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACScan, Becton Dickinson, Heidelberg, Germany). Briefly, viable cells were incubated with prime antibody for 1 h at room temperature. As a detection antibody, a rabbit anti-mouse Ig-FITC conjugate (81-6711, Invitrogen) was added for another 30 min. Cells were analyzed immediately after washing three times in PBS. To exclude unspecific binding of first and second antibodies, each incubation step was preceded by blocking the endothelial cell Fc-receptors with normal rabbit serum (dilution 1:5 *v*/*v*) for 20 min. The number of specifically stained cells was calculated by subtracting the number of cells stained with the negative control antibody, MPC11 (Enzo Life Sciences, IgG2b, catalog number: ADI-SAB-602PED), from the total number of antibody-stained cells. The relative mean fluorescence intensity values are calculated as averages of at least 3 independent experiments. For each sample 5,000 to 10,000 viable cells were analyzed. Viability of the cells was determined by negative propidium iodide staining and trypan blue exclusion.

Cytotoxicity assay

For complement-mediated cytotoxicity (CMCC), endothelial cells (3×10^4/well) in 96-well flat-bottomed plates (3072; Falcon Labware) were incubated with OxLDL or only cultured and labeled with 100 μl of 10 % FCS containing 5 μCi ^{51}Cr (Behringwerke) for the last 90 min at 37 °C. After three washes with medium 199, 100 μl of diluted antibodies was added. In addition to pooled atherosclerotic rat plasma Hsp70 autoantibodies (Ig^{AS}) and pooled control rat plasma Hsp70 autoantibodies (Ig^{Nor}), anti-Hsp70 antibodies, including mAb anti-human Hsp70 (SPA-810, Stressgen), mAb anti-rat Hsp70 (BD091), four control antibodies, mouse IgG (IgG^{mouse}, Abcam, ab37356), mAb anti-rat Factor VIII (Abcam, ab61390), mAb anti-rat α-actin (Boster, China), and mAb anti-rat CD3 Ab (Abcam, ab5690) were separately added and incubated for 7 h at 37 °C in the presence of guinea pig serum (50 μl, 1140; Behringwerke) as source of complement. Guinea pig serum was selected to be free of anti-Hsp70 Ab (ELISA, enzyme-linked immunosorbent assay). At the end of the incubation period, 100 μl of cold (4 °C) 10 % FCS was added, the plates were centrifuged at 800×*g* for 10 min at 4 °C, and 150 μl of supernatant was removed and radioactivity was measured in a gamma counter (Wizzard Automatic Gamma Counter; Wallac, Helsinki, Finland).

To determine antibody-dependent cellular cytotoxicity (ADCC), peripheral blood mononuclear cells (PBMC) were isolated from healthy rats by density centrifugation (Lymphoprep, density 1,083; Nycomed Pharmaceuticals Oslo, Norway) as described previously (Jurgens et al. 1989). In a procedure similar to that used to test antibody CMCC, PBMC rather than complement were added as effector in 10 % FBS at various concentrations. Diluted antibodies were then added as the CMCC test. Cell-specific release of radioactivity was calculated according to the formula: specific release=(release in the presence of antibodies−spontaneous release)/(maximal release−spontaneous release). Maximal release was

represented by radioactivity in the presence of 1 % Triton X-100. Spontaneous release was determined by the addition of effectors to the culture in the absence of antibodies and did not exceed 15 % of maximal release. To exclude any cytotoxic effect of OxLDL, spontaneous releases were separately measured for ox-pretreated and unpretreated cells and referred to as an experimental release of treated and untreated cells, respectively. Antibody preparations alone were demonstrated not to be cytotoxic for OxLDL-treated and untreated cells.

Statistical analysis

Values are expressed as medians and quartiles or mean± SEM when appropriate. The Mann–Whitney *U* test and ANOVA were used to test statistical significance. A value of $p<0.05$ was considered significant.

Results

Correlation between plasma anti-Hsp70 autoantibodies and OxLDL in atherosclerosis

Numerous studies have demonstrated that OxLDL can accelerate atherosclerosis and cause endothelial dysfunction. An elevated level of low density lipoprotein (LDL) cholesterol in plasma is a widely recognized risk factor for atherosclerosis (Jialal and Devaraj 1996). In this study, atherosclerosis was observed in all the SD rats in the atherosclerotic (AS) group at 10 weeks (10 w). At 10 w, the pathological changes that occurred in rats were very similar to those in humans, and plaques were observed in the aorta in the AS group (Fig. 1a). The level of plasma OxLDL in the AS group was significantly higher than those in the control group (Fig. 1b, $p<0.05$ compared with control), consistent with others (Vasankari et al. 2001). Different results show the different level of circulating anti-Hsp70 antibody in cardiovascular disease. In the present study, total levels of anti-Hsp70 autoantibodies (Igs) increased markedly in atherosclerosis (Fig. 1c). The level of total anti-Hsp70 autoantibody had a positive correlation with the level of OxLDL (Fig. 1d, $R^2=0.729$, $p<0.01$). In order to demonstrate the specificity of the humoral response, the isotypes of anti-Hsp70 autoantibody were determined. The level of IgG anti-Hsp70 autoantibody significantly increased in atherosclerosis along with less pronounced IgA, while the level of IgM also markedly increased (Fig. 1e, $p<0.05$ compared with the control). These results suggest that anti-Hsp70 antibody may be involved in the pathogenesis of atherosclerosis.

Hsp70 is present within membrane fraction

OxLDL, which was detected in the atherosclerotic plaque, causes endothelial activation, and dysfunction. Thus, OxLDL may have a toxic effect on endothelial cells depending on its concentration and the exposure time. RAECs were incubated with OxLDL (at different times and concentrations) in M199 medium. Membrane separation fraction was performed using viable untreated and treated cells. The membrane fractions from both control and OxLDL-treated cells were all positive for the γ-glutamyl-transferase activity (data not shown). The HSC70 protein was not detected in membrane fractions as shown in Fig. 2a,b,e (below). These data suggested that the membrane fractions were derived from the cell plasma. It has been reported that Hsp70 differentially localizes to the nucleus, to the cytosol and to the cell surface (Multhoff and Hightower 1996). To further investigate Hsp70 in membrane, membrane fractions were confirmed by western blotting. Hsp70 was only detected in membrane fraction from the cells incubated with 10–200 μg/ml OxLDL 12 h or with 20 μg/ml OxLDL 6 h later (Fig. 2a, b, above). The non-lethal OxLDL-dose parameters for adherent endothelial cells were defined by the ^{51}Cr release assay. The ^{51}Cr release of radioactivity in supernatant was not significantly increased at the concentration of 20 μg/ml OxLDL treatment for 12 h (Fig. 2c, d). If not otherwise indicated, OxLDL treatment was performed at 20 μg/ml OxLDL 12 h in M199 medium followed by recovery at 37 °C for 90 min. As shown in Fig. 2e, following treatment, the cytoplasmic amounts of Hsp70 in the control and OxLDL-treated cells were comparable. However, the endothelial membrane Hsp70 was only detectable in the membrane fraction of OxLDL-treated cells, but not in control cells.

Hsp70 is present on the endothelial cell surface

The preceding observations suggest that Hsp70 is capable of presenting within membrane fraction. We further investigated whether Hsp70 is present on cellular surface membrane by performing immunostaining and flow cytometry. After OxLDL treatment at 20 μg/ml for 12 h, cell viability was >98 % in both normal and treated cells (data not shown). OxLDL treatment at concentrations above 50 μg/ml markedly induced cell damage (Fig. 2c). Interestingly, surface staining was found on living endothelial cells treated at 20 μg/ml OxLDL and probed by BD091. This surface staining was clearly verified by fluorescence microscope (Fig. 3d). Very weak, if any, staining was seen on untreated cells probed with BD091 (Fig. 3c). Moreover, the SPA-810 failed to reveal the presence of Hsp70 on the cells, regardless of OxLDL treatment (Fig. 3a and b). Under the same

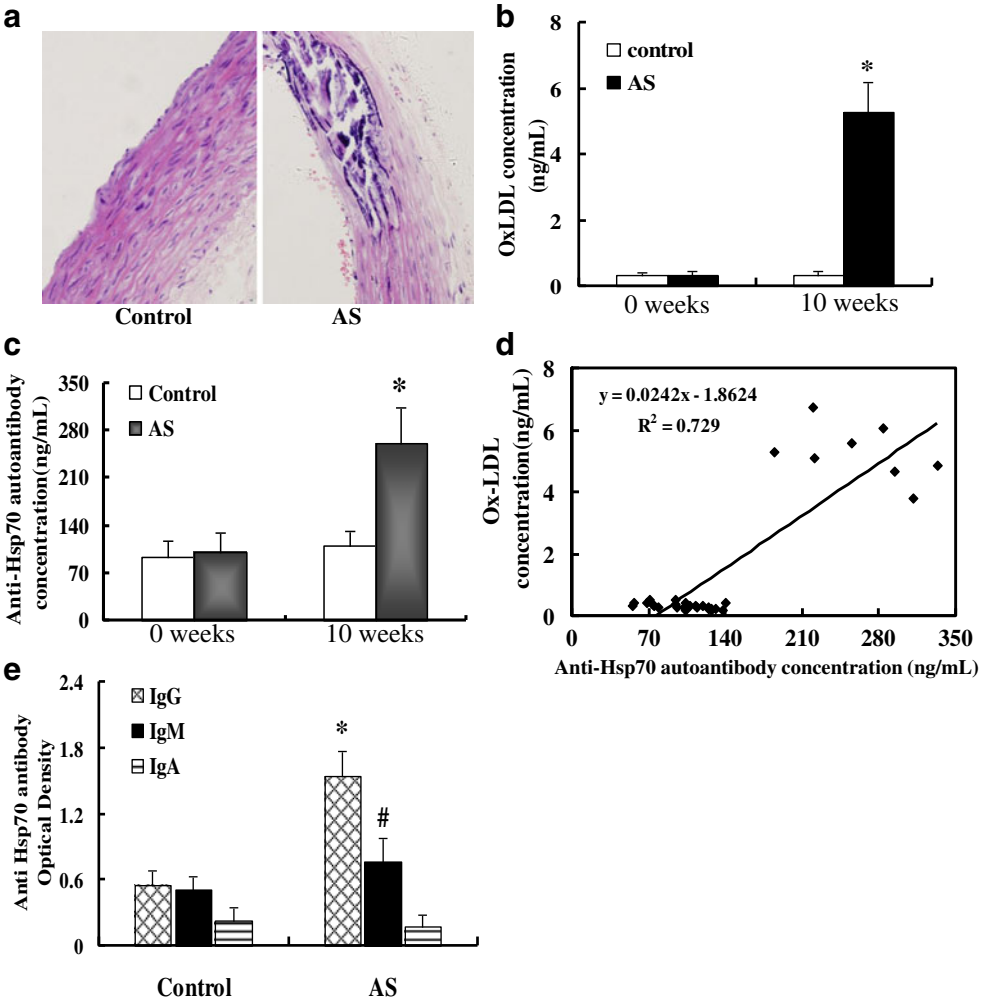

Fig. 1 Correlation between plasma anti-Hsp70 autoantibodies and OxLDL. The level of plasma OxLDL and anti-Hsp70 autoantibody in rat atherosclerosis on a high-cholesterol diet for 10 weeks. **a** The pathological changes of rat aortae (HE, ×200). **b** The level of OxLDL in plasma and **c** the concentration of anti-Hsp70 autoantibody were measured by ELISA as described in Materials and Methods. **d** Representative graph of the relationship between Hsp70 IgG autoantibody and OxLDL. **e** the levels of anti-Hsp70 autoantibody isotypes at 10 weeks. Values are means±SD from three independent experiments, $n=8$, $^{*}p<0.05$ compared with the control group

conditions, an IgG isotype-matched control antibody failed to reveal the presence of Hsp70 (data not shown).

Indirect immunofluorescence studies using Hsp70 specific MAbs (BD091, SPA-810) followed by FACScan analysis were performed with untreated and OxLDL-treated cells. As a result, treatment of the endothelial cells with OxLDL considerably increased the possibility of detection of Hsp70 on the cell surface by BD091. The percentage of Hsp70 positively stained cells following OxLDL (20 μg/mg) compared to control cells (non-OxLDL-treated) is given (Table 1). Expression was detectable on OxLDL-treated cells (27 %). While under identical OxLDL conditions, no significant induction of Hsp70 cell surface expression could be observed by another Hsp70 antibody (SPA-810, 2 %). To discount the possibility that small amounts of cytoplasmic Hsp70 released during cell lysis could have led to non-specific association of Hsp70 with the outer cell membrane, we performed co-incubation experiments followed by FACScan analysis. The results for the endothelial cells are summarized in Table 2. Untreated endothelial cells were incubated for 12 h with supernatants of lethally OxLDL-treated (200 μg/mg for 12 h) endothelial cells, nonlethally treated (20 μg/mg for 12 h), which contain Hsp70; no surface association of Hsp70 with the outer cell membrane could be found. Thus these observations suggest that Hsp70 is present on the surface of the endothelial cells after OxLDL treatment. We named this membrane Hsp70 for endothelial surface membrane Hsp70

CMCC and ADCC

The injury to endothelial cells was judged a primary event in the pathogenesis of atherosclerosis. Based on the observations described above, the cytotoxic potential of anti-Hsp70 antibody was determined on confluent endothelial cells labeled with ^{51}Cr. As shown in Fig. 4a, when cultured endothelial cells were treated with different concentrations of antibody, a specific dose-dependent release of radioactivity from OxLDL pretreated cells was found in the presence of anti-Hsp70 antibody (BD091) or Ig^{AS} and complement. This suggests that not all anti-Hsp70 antibodies could induce the ^{51}Cr release (SPA-810), but only specific epitope antibodies such as BD091 could do so. The release of ^{51}Cr is significantly different between OxLDL pretreated cells and

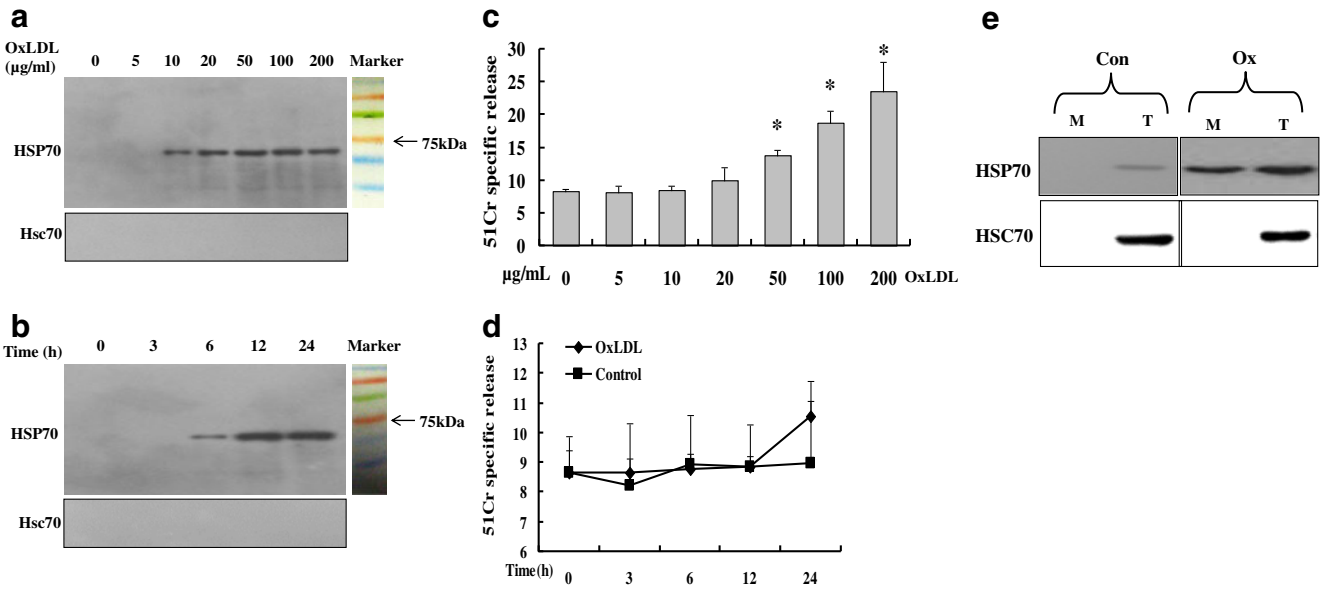

Fig. 2 Hsp70 is present in membrane fractions depending on OxLDL-treated. RAECs were maintained with OxLDL treated at 37 °C, recovered (37 °C for 90 min in M199 medium), and lysed in TNE buffer containing 1 % Triton X-100. Membrane fractions were isolated as described in "Materials and Methods". **a** RAECs were treated with 0-200 μg/mL for 12 h; **b** RAECs were treated with 20 μg/mL for 3, 6, 12, and 24 h. The presence of Hsp70, Hsc70 within membrane fraction was detected by Western blotting (**a**, **b**), whereas the supernatant ^{51}Cr release was determined in a gamma counter (**c**, **d**). After OxLDL treatment, cells were washed with medium 199 three times, and 5 μCi ^{51}Cr in 100 μL of medium 199 containing 10 % FCS was added to each well and incubated at 37 °C for 1.5 h. Cell culture supernatant was for radioactivity detection. **e** RAECs were incubated with 20 μg/mL OxLDL for 12 h, non-OxLDL-treated as control. The presence of Hsp70, Hsc70 in membrane fraction (*M*) and total cellular extracts (*T*) were detected by western blotting. Data are mean of three experiments. *Significant difference from controls ($p<0.01$)

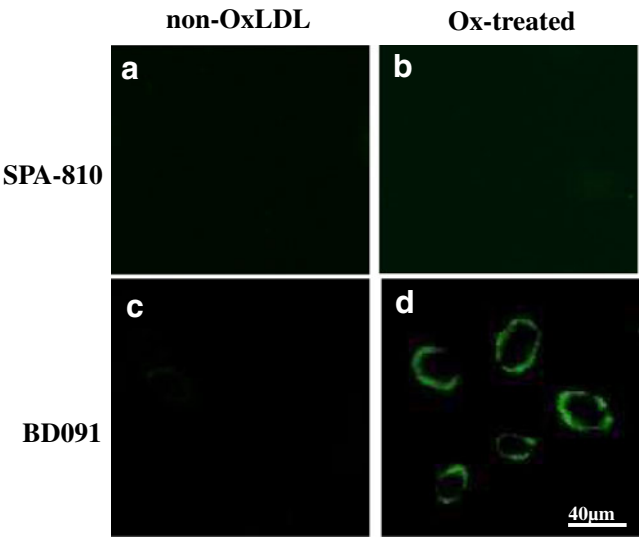

Fig. 3 Hsp70 is present on the surface of OxLDL-treated cells. RAECs were treated (20 μg/mL OxLDL treated for 12 h) or not with OxLDL at 37 °C, recovered for 90 min at 37 °C in M199 medium, incubated with Abs against Hsp70 (BD091 (*bottom panel*, **c**, **d**) or SPA-810 (*top panel*, **a**, **d**)) at 1 h at room temperature. After three washes with PBS, the cells were incubated with Ig-FITC conjugate for 30 min, fixed with absolute methanol for 5 min, rinsed, and embedded in *n*-propylgalat/glycerol. The green fluorescence shows the detection of Hsp70 on the cell surface

untreated cells ($p<0.01$), although an average of 11 % was also released from untreated cells in the presence of the anti-Hsp70 Ab (Fig. 4b). ^{51}Cr release was only slightly above background when labeled cells were incubated with Ig^{Nor}, mAb anti-Factor VIII, mAb anti α-actin, mAb anti-Hsp70 (SPA-810), or mAb anti-CD3 (Fig. 4b).

To determine the potential of anti-Hsp70 Ab to produce ADCC, OxLDL pretreated and untreated endothelial cells were incubated with the anti-Hsp70 Abs in the presence of normal rat PBMC. Figure 5a shows ^{51}Cr release at various effector-to-target ratios. Higher concentrations of effector cells, i.e., 50:1 and 100:1, entailed a significant degree of ADCC on OxLDL pretreated compared with untreated cells ($p<0.01$).

Table 1 Flow cytometric analysis of Hsp70 (anti-Hsp70 Mab, BD091 or SPA-810) cell surface expression on the endothelial cells, either untreated or subjected to OxLDL (20 μg/ml)

	% positively Hsp70 stained cells (average mean fluorescence intensity)	
	Non-OxLDL	OxLDL
BD091	2(35)	27(91)
SPA-810	1(37)	2(40)

OxLDL (20 μg/ml, 12 h; 37 °C, 90 min). Data are mean values from at least three independent experiments

Table 2 Flow cytometric analysis of Hsp70 (anti-Hsp70 Mab, BD091) cell surface expression on the endothelial cells after 20 μg/ml OxLDL treatment and after co-incubation of untreated the endothelial cells after with Hsp70 containing supernatants of lethally OxLDL-treated (200 μg/ml OxLDL) cells

Treatment					%positively stained endothelial cells(Hsp70)
Non-OxLDL	20 μg/ml OxLDL	The endothelial cells exposed to supernatants of lethally treated cells			
		Non-OxLDL	20 μg/ml OxLDL	200 μg/ml OxLDL	
+	—	+	—	—	0
+	—	—	+	—	0
+	—	—	—	+	1
—	+	—	—	—	28

Non-OxLDL (non-OxLDL-treated); 20 μg/ml OxLDL(20 μg/ml OxLDL treated for 12 h and recovery for 90 min); 200 μg/ml OxLDL(lethally treated, 200 μg/ml OxLDL treated for 12 h and recovery for 90 min). Data are mean values from three independent experiments

More than 52 % of total labeled ^{51}Cr was released from OxLDL pretreated endothelial cells by anti-Hsp70 antibodies (BD091, Ig^{AS}), while controls (Ig^{Nor}, mAbs anti-Factor VIII, anti-α-actin, SPA-810, or anti-CD3) induced <15 % of ^{51}Cr release from either OxLDL-treated or untreated cells (Fig. 5b). Thus, anti-Hsp70 antibody (BD091) or Ig^{AS} were able to lyse endothelial cells in vitro via either CMCC or ADCC.

Discussion

This study demonstrated that the level of plasma anti-Hsp70 autoantibody was significantly increased in atherosclerotic rats. These antibodies could react with endothelial surface membrane Hsp70 and could mediate endothelial cytotoxicity, demonstrating the possible involvement of humoral immune reaction to Hsp70 in the pathogenesis of atherosclerosis.

Previous studies have reported that elevated levels of Hsp antibodies are detected in patients with atherosclerosis (Xu et al. 1999; Pockley 2002). The levels of anti-Hsp70 autoantibody in cardiovascular disease were not the same in different studies. Our results show a high level of anti-Hsp70 autoantibody in atherosclerotic rats. In contrast, lower levels of anti-Hsp70 antibody were found in patients with coronary atherosclerosis, and acute coronary syndrome group (Zhang et al. 2011). It may be that, under different conditions, the autoantibodies present at different levels.

In this study, Hsp70 present on the surface of the endothelial cells treated by OxLDL with BD091 was clarified first time. The other anti-Hsp70 antibody (SPA-810), could not recognize the endothelial surface membrane Hsp70. This seems to suggest that different regions of the Hsp70

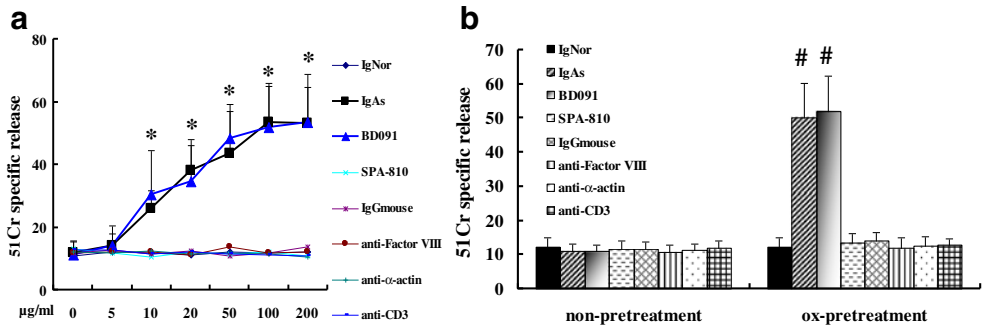

Fig. 4 Dose–response curves for CMCC. Confluent RAECs (0.5×10^6 cells/mL) in 96-well plates were treated (20 μg/mL for 12 h at 37 °C) or not with OxLDL, and recovered for 90 min at 37 °C. After three washes with medium 199, 5 μCi ^{51}Cr in 100 μL of medium 199 containing 10 % FCS was added to each well and incubated at 37 °C for 1.5 h. After two further washes, antibodies (*filled square* Ig^{AS}; *filled diamond* Ig^{Nor}; *filled star* IgG^{mouse}; mAb anti-Hsp70 (*filled upright triangle* BD091; *ex symbol* SPA-810); *filled circle* mAb anti-Factor VIII; *vertical line* mAb anti-α-actin; and *horizontal line* mAb anti-CD3) at indicated concentrations in 100 μL of medium were added, and incubated at 37 °C for 7 h in the presence of guinea pig serum as a complement source. After incubation, supernatant radioactivity was determined in a gamma counter. The values are means of three experiments, each performed in triplicate. *$p<0.01$ vs SPA-810; $^{\#}p<0.01$ vs nonpretreatment

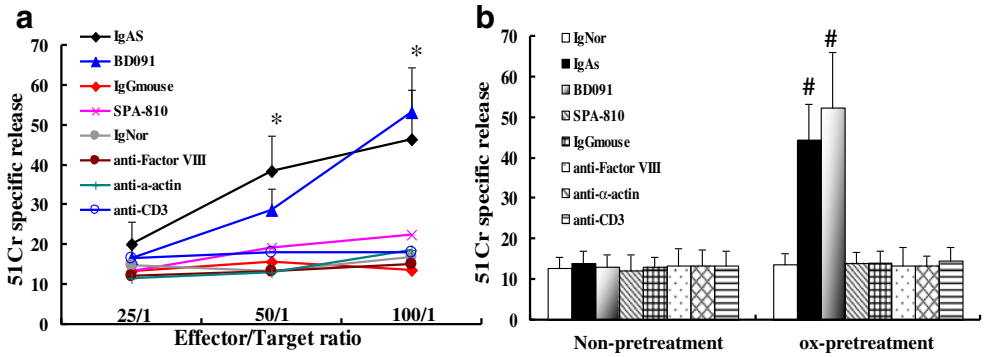

Fig. 5 Dose–response curves for ADCC. The procedures for OxLDL incubated and Ab treatment were the same as described in the legend for Fig. 4. As effectors normal rat PBMC were added to the culture instead of complement. Note increased 51Cr release at higher effector/target cell ratios (**a** 20 μg/mL OxLDL pretreated rat aortic endothelial cells, **b** OxLDL-treated and untreated endothelial cells as effector/target was 50/1). *Black filled diamond* Ig^{AS}; *gray filled circle* Ig^{Nor}; mAb anti-Hsp70 (*filled upright triangle* BD091; *ex symbol* SPA-810); *brown filled circle* mAb anti-Factor VIII; *vertical line* mAb anti-α-actin; *open circle* mAb anti-CD3; *red filled diamond* IgG^{mouse}. *Significant difference from untreated cells, $*p<0.01$ vs SPA-810; $^{\#}p<0.01$ vs nontreatment

were exposed to the extracellular. Higher Hsp70 expression was reported in intracellular endothelial cells after treatment with OxLDL (Zhu et al. 1994). The mechanism for the Hsp70 translocation to membrane after treatment with OxLDL was interesting. Transport of other proteins across lipid membranes is one of the major tasks of Hsp70. Although lacking a classical consensual transmembrane sequence, it is conceivable to assume that cytosolic Hsp70 is transported to the plasma membrane in concert with other proteins possessing transmembrane domains to fulfill shuttle functions or through a direct interaction of Hsp70 with lipid components under definite conditions. Given that high salt and major changes in pH fail to eliminate Hsp70 from the plasma membrane of the endothelial cells (unpublished observations), an electrostatically driven binding of Hsp70 to protein-based membrane components appears to be unlikely. It is likely that Hsp70 interacts with fatty acids of the plasma membrane, as has been proposed by Hightower and Guidon (1989). Studies have shown that Hsp70 could be released to the supernatants of viable tumor cells (Guzhova et al. 2001; Triantafilou et al. 2002). Our results show that the cell surface membrane Hsp70 was not from supernatant Hsp70 by using co-incubation experiment. Other researchers have shown that members of the Hsp70 family preferentially interact with artificial liposomes in the presence of phosphatidylserine (Arispe et al. 2002; Arispe et al. 2004). Gehrmann confirmed that Gb3 act as an interaction partner for Hsp70 in the plasma membrane of tumor cells (Gehrmann et al. 2008). These phenomena were observed in tumor cells. Whether the same mechanism is true for Hsp70 translocation to endothelial surface membrane needs to be further clarified.

OxLDLs may alter the fragile balance between survival and death in vascular cells, thereby leading to atherosclerosis. Sanson et al. have reported that OxLDLs induced the unfolded protein response and triggered endoplasmic reticulum stress in human vascular cells (Sanson et al. 2009). Intracellularly, Hsp70 perform a variety of chaperoning functions. Thus, Hsp70 may bind to and stabilize nonnative conformations of other proteins and inhibit aggregation of unfolded proteins or enable translocation across the endothelial cell membranes after OxLDL treatment. This validates our findings. The amount of endothelial surface membrane Hsp70 was dependent on OxLDL treatment. This may be the new mechanism for Hsp70 translocation to membrane after OxLDL treatment in the endothelial cells.

Accumulating evidence indicates that antibodies and complement are involved in atherogenesis (Salonen et al. 1992). Muscari et al. (1988) reported that the levels of serum Hsp70 IgA of the patients with severe atherosclerosis were significantly higher than those of the control group. Our laboratory studies found that Ig extract from atherosclerotic plaque could react with Hsp70 protein (data not shown). George et al. (2001) observed that Hsp70 IgG deposition in rat carotid arteries after balloon injury may have functional effects on the endothelial cells. Thus, the questions that arise are whether anti-Hsp70 antibody (BD091) can bind on the endothelial surface membrane Hsp70 and induce endothelial cell damage. In the presence of complement or PBMC, BD091 and Ig^{AS} significantly induced endothelial cells injury in the OxLDL-treated group. Meanwhile in the non-OxLDL-treated group, BD091 and Ig^{AS} had no effect on endothelial cells. It is very interesting that the other Hsp70 antibody (SPA-810), which recognized the other epitope of Hsp70 did not have the same effect as BD091. Anti-Hsp70 antibody (BD091) could induce endothelial cells injury by ADCC or CMCC.

The endothelial surface membrane Hsp70 was observed when treated with 20 μg/ml OxLDL for 12 h. Under these conditions, the endothelial cells were not obviously

damaged (more than 98 % of the cells excluded trypan blue and propidium iodide). Thus, we suppose that, in the earlier stage of atherosclerosis, low concentrations of OxLDL could induce Hsp70 translocation to the membrane of endothelial cells. Hsp70 autoantibodies react with endothelial surface membrane Hsp70 and induce the endothelial cell damage. This may be an evidence of a role for both anti-Hsp70 antibody and endothelial surface membrane Hsp70 in atherosclerosis.

In conclusion, the anti-Hsp70 antibody could induce endothelial cell injury through ADCC or CMCC via the cell surface membrane Hsp70. The anti-Hsp70 antibody might play a role in the induction of endothelial cell injury correlating OxLDL. Why endothelial cells present Hsp70 on their cell surface remains to be elucidated.

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