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ORIGINAL ARTICLE

Relationship between plasma cholesterol, von Willebrand factor concentrations, extent of atherosclerosis and antibody titres to heat shock proteins–60, –65 and –70 in cholesterol–fed rabbits

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Summary

Epidemiological studies have shown an association between atherosclerosis, Heat shock protein (Hsp) expression, and Hsp antibody titres. We aimed to investigate the time course of appearance of Hsp-60, -65 and -70 antibodies in the cholesterol-fed rabbit and to relate antibody titres to serum concentrations of von Willebrand factor (vWF), a marker of endothelial injury. Rabbits were fed with 0.25–1.0% cholesterol diet for 13 weeks. Plasma levels of anti Hsp-60, -65 and -70 IgG titres, were measured using in-house enzyme-linked immunosorbent assays (ELISAs) together with plasma vWF concentrations. Plasma titres of anti-Hsp-60, -65 and -70 antibodies were all significantly increased by weeks 5, 7 and 9 following commencement of the experimental diet compared with baseline ($P < 0.05$ for all). In non-cholesterol-fed rabbits, plasma levels of anti-Hsp titres were unchanged over this period. Increased plasma vWF concentrations were also found in the cholesterol-fed rabbits, reaching a maximum at approximately week 8, and falling thereafter. Furthermore, plasma vWF concentrations at 13 weeks correlated strongly with antibody titres to all three Hsps ($r = 0.90$, $P = 0.002$; $r = 0.80$, $P = 0.017$; $r = 0.86$, $P = 0.006$ for Hsp 60, -65 and -70 respectively) and titres were also strongly correlated with final plasma cholesterol concentrations in cholesterol-fed animals ($r = 0.95$, $P = 0.002$; $r = 0.8$, $P = 0.001$; $r = 0.84$, $P = 0.01$ respectively). In cholesterol-fed rabbits, antibody titres to Hsp-60, -65 and -70 appear to rise in association with a marker of endothelial injury, peaking at approximately the same time (8 weeks) after starting a high cholesterol diet.

Keywords
cholesterol-fed rabbit, endothelium, Heat shock protein-60, -65 and -70, vWF

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Cells respond to a variety of environmental stresses by expressing several families of proteins, collectively called Heat shock proteins (Hsps) (Morimoto 1993; Udelsman *et al.* 1993). These proteins are molecular chaperones, involved in the process of renaturation of other proteins. However, they may themselves be damaged and it has been proposed that they may then become antigenic (Lamb *et al.* 2003).

Clinical studies have reported positive associations between antibody titres to several Hsps and extent of cardiovascular disease (CVD) (Burian *et al.* 2001). We have previously shown that in dyslipidaemic and obese individuals there are elevated titres to Hsp-60, -65 and -70, perhaps indicating a heightened state of immunoactivation associated with these conditions (Ghayour-Mobarhan *et al.* 2005b). We have also reported that titres of antibodies recognizing these Hsps were not related to classical coronary risk factors (Ghayour-Mobarhan *et al.* 2005a), and that they may be affected by dietary constituents such as antioxidants (Ghayour-Mobarhan *et al.* 2005a). Furthermore, stimulation of an immune response to certain Hsps appears to enhance atherogenesis in the cholesterol-fed rabbit model (Lamb & Ferns 2002).

In the response to injury hypothesis (Ross 1999) it was proposed that endothelial injury is the initiating event in atherogenesis. Because endothelial cells are located at the interface between the blood and artery wall, they are also the first cells to encounter the burden of an insult.

Von Willebrand Factor (vWF) is a procoagulant glycoprotein derived from endothelial cells and platelets that is involved in platelet adhesion and aggregation at sites of vascular injury, and serves as a carrier for the coagulation factor VIII (Ewenstein 1997). A raised plasma concentrations of soluble vWF is also considered to be index of endothelial cell activation and/or dysfunction (Ewenstein 1997), and an index of endothelial damage in vascular disease (Boneu *et al.* 1975). It has been postulated that the plasma membrane of damaged endothelial cells leaks vWF, leading to an increase of the plasma levels of this protein (Stehouwer *et al.* 1995).

Several studies have found that plasma vWF concentrations are high in clinical situations characterized by vascular injury associated with endothelial damage (Kahaleh *et al.* 1981; Cucuianu *et al.* 1983). Furthermore, plasma vWF concentrations are also elevated in situations in which atherosclerosis is present at its very earliest stages (e.g. children with risk factors) (Wojakowski & Gminski 2001), or before morphological evidence of injury in a rat model of endothelial injury (Newsholme *et al.* 2000). It has also been shown that in rabbits fed 0.3% cholesterol for 26 weeks, the increased expression of vWF is reversible on cholesterol withdrawal, which is also associated with normalization of endothelial morphology (De Meyer *et al.* 1999).

The aim of this present study was to investigate the temporal relationship between the appearance of anti-Hsps titres and vWF in a well-characterized model of atherosclerosis, the cholesterol-fed rabbit.

Material and methods

Rabbit colonies

Juvenile New Zealand White rabbits (10 weeks old) weighing approximately 2.0 kg were housed in the experimental biology unit at the University of Surrey, Guildford in accordance with Home Office regulations. Food and water was allowed *ad libitum*.

Cholesterol feeding

Each experimental group consisted of eight rabbits that were either fed a normal chow diet, or a diet containing 0.25–1% (w/w) cholesterol diet (Special Diet Services, Whitham, Essex, UK). The cholesterol content of the diet was individualized for each rabbit to maintain the plasma cholesterol levels between 20 and 30 mmol/l (Lamb *et al.* 1999) .

Blood sampling

Blood was collected from an ear vein prior to immunization and at fortnightly intervals during the experimental period. Venous blood was collected into heparinized containers and plasma obtained by centrifugation at 1500 ***g*** at 4 °C. Plasma cholesterol levels were measured using a Boehringer Accutrend meter with Accutrend test strips (Boehringer Mannheim, Lewes, East Sussex, UK).

Anti-Hsp antibody measurement

Plasma antibody titres to Hsps were measured using an ELISA. Microtitre plates (96-well, Nunc Immunoplate Maxisorp; Life Technologies, UK) were coated with 10 ng recombinant human heat shock protein-60, Hsp-65, Hsp-70 (Sigma, Poole, Dorst, UK) in phosphate-buffered saline (PBS) per well for 18 h at 4 °C under humidified conditions. The wells were washed three times in wash buffer (PBS containing 0.05% Tween 20). Non-specific binding was reduced by blocking each well with Superblock™ (Pierce & Warriner, Chester, Cheshire, UK) for 1 h at 37 °C. Wells were washed three times in wash buffer. Plasma was diluted 1:100 with PBT (PBS containing 0.1% Tween-20 and 1% bovine serum albumen) and 100 µl/well incubated for 30 min at 37 °C. After washing, bound antibodies were detected using Vector

anti-rabbit Elite ABC peroxidase kit (Vector Laboratories, Peterborough, UK) according to manufacturers instructions.

o-Phenylenediamine (0.04%) (Sigma) was dissolved in 0.05 M citrate/0.1 M phosphate buffer pH 5 containing 10 μl H_2O_2 per 25 ml. Substrate (100 μl/well) was incubated at room temperature for 5 min and the reaction terminated by adding 50 μl 3 M HCl. Optical density at 492 nm was measured using a Labsystems iEMS Reader MF microtitre plate reader with Genesis 2 software (Life Sciences, Basingstoke, Hampshire, UK).

Plasma von Willebrand factor ELISA

Plasma von Willebrand factor (vWF) was determined by sandwich ELISA. Microtitre plates (Nunc Maxisorp, Nunc) were coated with a polyclonal goat anti-vWF capture antibody (Cedarline Laboratories, Hornby, ON, Canada) in PBS per well for 18 h at 4 °C under humidified conditions. The wells were washed and blocked as before. Plasma was diluted 1:100 with PBT and 100 μl/well incubated for 30 min at 37 °C. After washing, bound antibodies were detected using peroxidase- conjugated polyclonal goat anti-vWF antibody (Cedarline Laboratories). The substrate was prepared and developed as before.

Quantification of lesions

Longitudinal halves of aortae were rinsed in 80% propan-2-ol and stained for 90 s in 80% propan-2-ol containing 2% (w/v) oil red O (Sigma). Aortae were rinsed in 80% propan-2-ol containing 2% (w/v) oil red O followed by de-staining in 80% propan- 2-ol and PBS as described previously (Rutherford *et al.* 1997). The sections were pinned out on a cork board and en face images acquired using a JVC CCD camera. The area staining positively with oil red O was quantified using QWin 550C image analysis software (Leica Microsystem, Cambridge, UK) and expressed as a percentage of the total area analysed.

Aortic segments were taken at the level of the first intercostals branches and embedded in paraffin. Five micron sections were stained with Verhoff-Van Geisen (VVG) stain. The extent of atherosclerosis was measured by histological determination of the intimal/medial ratio using a Leica DLMB microscope equipped with a ×10 objective, JCV CCD camera and QW in image analysis system (Leica Microsystems).

Statistics

Statistical analyses were carried out using the statistical package MINITAB Release 13 (Minitab Inc., 3081 Enterprise Drive, State College, PA16081-3008, USA). Quantitative data were assessed for normality using the Kolmogorov–Smirnov tests. Normally distributed data were analysed using one-way of variance analysis (ANOVA). Non-normally distributed data were normalized using log-transformed and these log-transformed data were analysed using one-way analysis of variance. *Post hoc* tests (Fisher's method) were used after using one-way analysis of variance. The correlation between anti-Hsps and variances such as total cholesterol, intima: media area ratio and vWF was assessed by using Pearson correlation.

Results

Plasma anti-Hsp antibody levels

Mean plasma anti-Hsp-60, 65, and 70 titres rose rapidly and became significantly higher than baseline by week 5, remaining significantly higher at 7 and 9 weeks, after starting the high cholesterol diet. Anti-Hsp-60 antibody titres were significantly higher at weeks 5 ($P < 0.05$), 7 and 9 ($P < 0.01$) compared to baseline (Figure 1a). Similar results were observed for Hsp-65 and Hsp-70 with $P = 0.001$ and 0.0016 respectively (Figure 1b,c). In the control rabbits, that received the normal chow diet, anti-Hsps titres did not change significantly between baseline and week 13.

Plasma level of vWF

In the cholesterol fed rabbits, mean plasma vWF (Figure 2) rose gradually up to week 7 when concentrations reached a peak, gradually falling thereafter. The peak in mean anti-Hsp titres coincided with the appearance of high level vWF in the plasma. In the control rabbits, that received the normal chow diet, plasma vWF titres did not change significantly between baseline and week 13.

Effect of cholesterol on atherosclerosis in thoracic aorta of cholesterol-fed rabbits

Not surprisingly, the percentage of the aorta staining positively for lipid using oil red O was significantly greater in the aortae from rabbits that were maintained on a cholesterol enriched diet compared with the normal chow fed animals ($P < 0.05$).

The relationship between the extent of atherosclerosis, plasma cholesterol concentrations and Hsp antibody titres

In the cholesterol fed rabbits, plasma anti-Hsp-60 and -70 titres were significantly correlated with the extent of

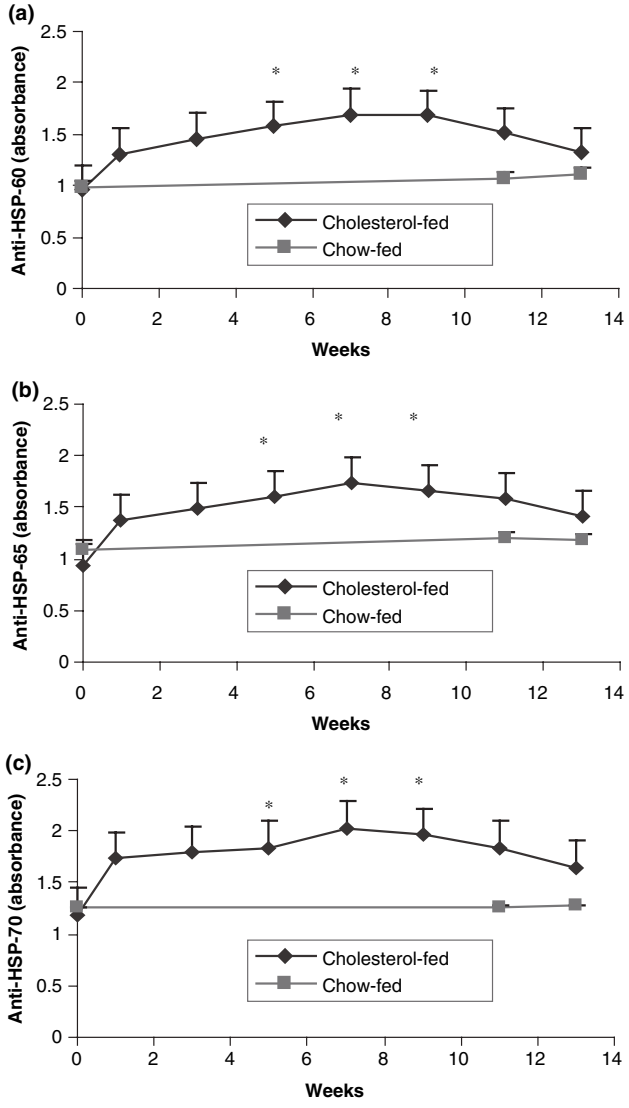

Figure 1 Time course for changes in plasma anti-Hsp-60, -65 and -70 titres in normal chow and cholesterol-fed rabbits. (a) Antibody titres to Hsp 60 were significant higher for cholesterol compared to normal chow-fed animals during the experimental period ($P = 0.0013$, by ANOVA), also being significantly higher at weeks 5 ($P < 0.05$), 7 and 9 ($P < 0.01$) compared with baseline. (b) Antibody titres to Hsp 65 were significant higher for cholesterol compared to normal chow-fed animals during the experimental period ($P = 0.001$, by ANOVA), also being significantly higher at weeks 5 ($P < 0.05$), 7 and 9 ($P < 0.01$) compared with baseline. (c) Antibody titres to Hsp 70 were significant higher for cholesterol compared with normal chow-fed animals during the experimental period ($P = 0.0016$, by ANOVA), also being significantly higher at weeks 5 ($P < 0.05$), 7 and 9 ($P < 0.01$) compared with baseline.

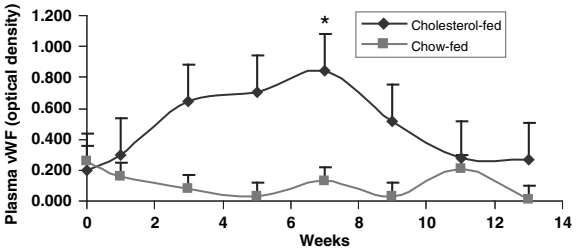

Figure 2 Time course of changes in plasma von Willebrand Factor (vWF) concentrations in normal chow and cholesterol-fed rabbits. Antibody titres to vWF were significant higher for cholesterol compared to normal chow-fed animals during the experimental period ($P < 0.001$, by ANOVA) also being significantly higher at week 7 $P < 0.01$ compared with baseline.

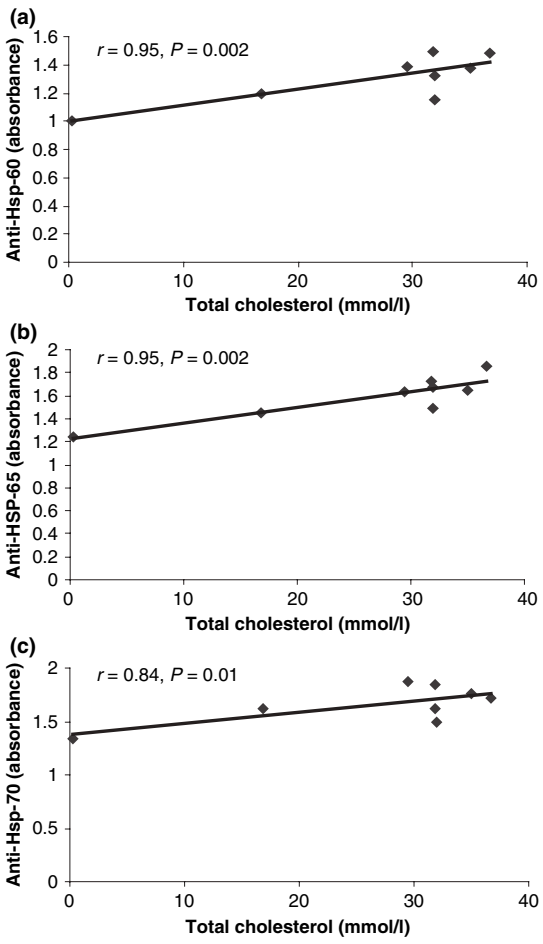

Figure 3 Relationship between plasma levels of anti-Hsp antibody titres and plasma cholesterol level. (a) Relationship between plasma levels of anti-Hsp-60 antibody titres and plasma cholesterol level. (b) Relationship between plasma levels of anti-Hsp-65 antibody titres and plasma cholesterol level. (c) Relationship between plasma levels of anti-Hsp-70 antibody titres and plasma cholesterol level.

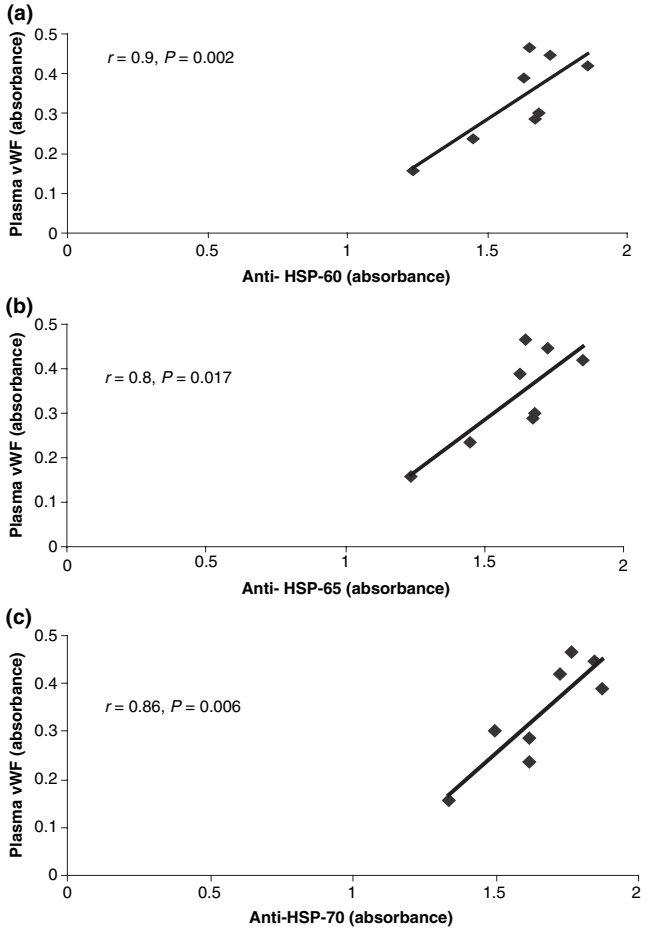

Figure 4 Relationship between plasma levels of anti-Hsp antibody titres and plasma vWF level. (a) Relationship between plasma levels of anti-Hsp-60 antibody titres and plasma vWF level. (b) Relationship between plasma levels of anti-Hsp-65 antibody titres and plasma vWF level. (c) Relationship between plasma levels of anti-Hsp-70 antibody titres and plasma vWF.

atherosclerosis as assessed by intima:media area ratio ($r = -0.84$, $P = 0.018$; $r = -0.84$, $P = 0.019$ respectively), and although plasma antibody titres to anti-Hsp 65 antibody were also associated with the intima: media area ratio ($r = -0.69$), this failed to reach statistical significance ($P = 0.08$).

Plasma anti-Hsp-60, -65 and -70 titres were also strongly associated with integrated plasma cholesterol concentrations ($r = 0.95$, $P = 0.002$; $r = 0.8$, $P = 0.001$; $r = 0.84$, $P = 0.01$ respectively, Figure 3a–c). Furthermore, plasma titres of anti-Hsp-60,-65 and -70 were also positively associated with the concentrations of plasma vWF ($r = 0.9$, $P = 0.002$; $r = 0.8$, $P = 0.017$; $r = 0.86$, $P = 0.006$ respectively, Figure 4a–c).

Discussion

There have been several epidemiological studies showing a positive association between peripheral blood concentrations of Hsp-60/-65 and extent of atherosclerosis (Zhu *et al.* 2001; Veres *et al.* 2002; Pockley *et al.* 2003). We have previously reported that following immunization with BCG, titres to Hsp 65 are associated with the extent of atherosclerosis in the cholesterol-fed rabbit model, and have discussed the mechanisms by which this may occur (Lamb *et al.* 1999). In this present study, we have investigated the time course of appearance of antibody titres to Hsp-60, -65 and -70 in the cholesterol-fed rabbit, relating the appearance of these antibodies to the appearance of a marker of endothelial injury, vWF, and the extent of atherosclerosis present in the aorta, assessed as the intima:media ratio.

Cholesterol feeding is associated with an anti-Hsp antibody response and a rise in vWF indicating endothelial injury

We found that antibody titres to Hsp-60, -65 and -70 rose soon after initiating a high cholesterol diet in the rabbit. In this model, using a target cholesterol of approximately 20 mmol/l, antibody titres to the Hsps reached a peak between 6 and 8 weeks after starting the atherogenic diet. No changes were observed in the animals fed a normal chow diet.

A significant increase above baseline values was also seen for mean plasma concentrations of vWF. This was observed from the first week of starting the high cholesterol diet, reaching a peak between weeks 7–8. Thereafter there was a gradual decline in plasma vWF concentrations.

The peak in plasma anti-Hsp-60, -65 and -70 titres coincided with the peak in plasma vWF concentrations. This temporal relationship suggests that endothelial injury may be associated with an enhanced expression of Hsp-60,-65 and -70, and that this may in turn lead to the antibody response. Another possibility is that an autoimmune response directed against the Hsps, mediates the endothelial injury.

Hsp antibody titres are related to plasma cholesterol and von Willebrand factor concentrations, and extent of aortic atherosclerotic lesions

We have previously reported (Lamb & Ferns 2002) that plasma antibody titres to Hsp-60 are strongly associated with the degree of subsequent lesion formation in the cholesterol-fed rabbit. The present data are consistent with these

findings. We found positive associations between anti-Hsp titres and plasma cholesterol concentrations in the cholesterol fed rabbits. These associations may be due to a combination of increased expression of the Hsps and enhanced immune response, both of which are associated with the atherogenic process. An increased expression of Hsps on the endothelium during atherogenesis has been reported previously (Khan *et al.* 1998; Lamb & Ferns 2002). We have also found a good correlation between the extent of atherosclerotic lesion formation in the aortae of cholesterol-fed rabbits and Hsp antibody titres. This was particularly the case for antiHsp-60 and -70 titres. Previous epidemiological studies have reported an association between Hsp antibody titres and the degree of extant atherosclerosis, and subsequent cardiovascular events (Xu *et al.* 1993, 1999; Herz *et al.* 2006).

Possible limitation of vWF as a marker of endothelial damage

Elevated plasma levels of vWF have been reported in several conditions associated with endothelial injury, or activation, including early atherosclerosis in man and rabbits (Wojakowski & Gminski 2001; De Meyer *et al.* 1999). It has been argued that elevations of vWF may not necessarily reflect gross endothelial damage or injury, but may better described as a marker of endothelial perturbation (Mannucci 1998). The distinction between endothelial injury, dysfunction and perturbation is a difficult one to elucidate, and in any case is likely to be within a spectrum of graded, progressive change associated with endothelial insult. Nevertheless, it appears from our study that early during the evolution of atherosclerosis in our experimental model, there is the appearance of a marker of endothelial dysfunction and antibodies to several Hsps thought to be involved in atherogenesis.

In conclusion, we have shown that vWF and antibodies to Hsp-60, -65 and -70 all rise rapidly during early atherogenesis, and are associated with the extent of atherosclerotic lesion formation in a rabbit model of atherosclerosis.

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