

Cross-Reactive B-Cell Epitopes of Microbial and Human Heat Shock Protein 60/65 in Atherosclerosis

Hannes Perschinka, Manuel Mayr, Gunda Millonig, Christina Mayerl, Ruurd van der Zee, Sandra G. Morrison, Richard P. Morrison, Qingbo Xu, Georg Wick

Objective—Growing evidence suggests that immune reactions to heat shock protein 60 (HSP60) are involved in atherogenesis. Because of the high phylogenetic conservation between microbial and human HSP60, bacterial infections might be responsible for breaking the tolerance to self-HSP60, which is expressed on the surface of stressed arterial endothelial cells.

Methods and Results—We purified serum antibodies to *Escherichia coli* HSP60 (GroEL), the 60-kD chlamydial HSP, and HSP65 of *Mycobacterium tuberculosis* by affinity chromatography from clinically healthy subjects with sonographically proven carotid atherosclerosis. Reactivity of the purified antibodies with overlapping human HSP60 peptides was measured, and 8 shared common epitopes, recognized by all anti-bacterial HSP60/65 antibodies, were identified. Antisera specific for these cross-reactive epitopes were produced by immunizing rabbits with peptides derived from human HSP60. By immunohistochemistry, the epitopes were found to be present in the arterial wall of young subjects during the earliest stages of the disease.

Conclusions—Antibodies to microbial HSP60/65 recognize specific epitopes on human HSP60. These cross-reactive epitopes were shown to serve as autoimmune targets in incipient atherosclerosis and might provide further insights into the mechanisms of early atherogenesis. (*Arterioscler Thromb Vasc Biol.* 2003;23:1060-1065.)

Key Words: epitopes ■ autoimmunity ■ atherosclerosis ■ heat shock protein 60 ■ aging

The current theory of atherosclerosis suggests that the first stages can be described as an inflammatory process in the arterial wall.¹ However, the initial triggering event and the involved (auto)antigens still remain controversial. Heat shock proteins of the 60-kDa family (HSP60) are among 1 of the most highly conserved families of proteins.² Prokaryotic HSP60s have homology >97% at their protein levels, whereas prokaryotic and human HSP60 (hHSP60) have >70% amino acid sequence homology. Although microbial HSP60s serve as major antigens in protection from and pathogenesis of infectious diseases, several autoimmune disorders, like rheumatoid arthritis, systemic sclerosis, psoriasis, Kawasaki disease, and Behcet's disease, are believed to be triggered by shared B- and T-cell epitopes that are cross-reactive between eukaryotic and prokaryotic HSP60.³ HSP60 can be found not only in mitochondria but also on the surface of stressed eukaryotic cells.^{4–6} Because chronic bacterial infections are known to be a risk factor for atherosclerosis,^{7,8} immunologic cross-reactions between bacterial HSP60 and hHSP60, which is expressed on the surface of stressed arterial cells, might be involved in atherogenesis. In a prospective,

longitudinal study, we demonstrated that elevated levels of anti-mycobacterial HSP65 (mHSP65) antibodies, cross-reacting with hHSP60, served as a prognostic marker for the incidence, prevalence, severity, and progression of carotid atherosclerosis in a clinically healthy population.^{9,10} Those results have subsequently been confirmed by others.^{11,12} Recent evidence corroborates that serum antibody reactivity to hHSP60 is also correlated with atherosclerosis.^{13–15}

We have demonstrated that purified human antibodies directed against different bacterial HSP60s are cross-reactive with hHSP60 and induce cytotoxicity through complement activation or antibody-dependent cellular cytotoxicity on stressed but not unstressed endothelial cells and macrophages.^{16–18} Animal models, in which early atherosclerotic lesions could be induced in normocholesterolemic rabbits and in LDL-receptor-deficient mice by immunization with recombinant mHSP65,^{19,20} gave further support to this concept. Interestingly, a *Chlamydia*-specific T-cell clone, isolated from an atherosclerotic plaque, was shown to be reactive with an epitope shared between chlamydial HSP60 (chHSP60) and hHSP60.²¹

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From the Institute for Biomedical Aging Research (H.P., M.M., G.M., Q.X., G.W.), Austrian Academy of Sciences, Innsbruck, and the Institute of Pathophysiology (H.P., C.M., G.W.), University of Innsbruck Medical School, Innsbruck, Austria; the Institute of Infectious Diseases and Immunology (R.v.d.Z.), Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands; and the Department of Microbiology (S.G.M., R.P.M.), Montana State University, Bozeman, Mont.

Correspondence to Prof Dr Georg Wick, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Peter-Mayr-Strasse 4b, 6020 Innsbruck, Austria. E-mail Georg.Wick@oeaw.ac.at

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We have previously reported on 3 dominant epitopes of mHSP65 that are recognized by serum antibodies from subjects with atherosclerosis.²² Interestingly, 2 of those seemed to associate into a single conformational structure, as determined by computer-assisted localization on an hHSP60 structural model.²³ The aim of the present study was to identify linear cross-reactive B-cell epitopes shared among HSP60s from *Escherichia coli*, *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and humans.

Methods

Purification of Serum Anti-HSP60 Antibodies

Blood samples were taken from participants in the Bruneck study, a large, population-based study of atherosclerosis.²⁴ Antibodies against mHSP65 were determined by ELISA by following an established protocol.⁹ For purification of anti-HSP antibodies, sera from 5 subjects with titers $\geq 1:1280$ and sonographically proven atherosclerotic lesions in the carotid artery were pooled. Affinity chromatography of serum was performed by using a previously described method.¹⁶ In brief, pooled sera was heat-inactivated, and immunoglobulins were precipitated by using a standard ammonium sulfate procedure. The precipitate was suspended in PBS (pH 7.2) and allowed to run through a chromatography column loaded with 2 mL agarose gel beads (Affi-Gel 15, Bio-Rad) coupled with 3 mg recombinant mHSP65, GroEL (Boehringer), or chHSP60. For each protein, 2 different serum pools were used. After washing away unbound proteins, specific immunoglobulins were recovered by elution with 20 mmol/L HCl. Fractions containing antibodies were immediately neutralized, pooled, dialyzed against PBS at pH 7.2, and adjusted to the original serum volume. Titers of purified serum anti-HSP antibodies were similar to those of the original serum pool ($\geq 1:1280$), whereas unbound immunoglobulins had no measurable HSP antibody titer.

Epitope Mapping With Spot Membranes

One hundred thirteen synthetic 13-mer peptides, comprising the whole sequence of hHSP with an 8-amino acid overlap, were used for epitope mapping. Peptides were immobilized on a nitrocellulose spot membrane with a (β -Ala)₂ spacer (Sigma-Genosys). After being blocked with 2.5% BSA (Sigma) in PBS overnight, the membrane was probed with affinity-purified human anti-HSP60 antibodies diluted 1:800 in PBS. Antibody binding was detected by incubation with peroxidase-conjugated rabbit anti-human immunoglobulins (P212, diluted 1:3000 in PBS; DAKO), followed by extensive washing with PBS/0.05% Tween 20 and visualization with an enhanced chemiluminescence kit (Amersham Pharmacia). The blots were scanned, and the derived signals were densitometrically quantified with ScanPack 2.0 software. For each anti-bacterial HSP60 antibody, a new membrane was used.

Peptide Synthesis

Identified epitopes (13 or 18 amino acids) were synthesized by using the concept of multiple antigenic peptides (MAPs).²⁵ In brief, 200 mg of a branched lysine core (Fmoc8-Lys4-Lys2-Lys-Ala-Novasyn PA80, 0.49 mmol \cdot L⁻¹ \cdot g⁻¹; NovaBiochem) was deprotected and coupled with a 4-fold excess of Fmoc-protected amino acids (NovaBiochem) in a continuous-flow peptide synthesizer under standard Fmoc chemistry conditions. Progress of the reaction was followed by monitoring the release of the cleaved Fmoc-protecting group at 310 nm, and a step was repeated when coupling efficiency was found to be $< 95\%$. Between the coupling steps, unreacted amino groups were capped with acetic anhydride (Sigma). After completion of the reaction, acidic hydrolysis with trifluoroacetic acid (Sigma) cleaved the MAP from the resin and removed the protecting groups from the side chains. The MAPs were recrystallized twice from diethyl ether/water. The recovery of pure product was between 65% and 80% of theoretical yield for all peptides.

Epitope-Specific Antibody Production and Purification

For each peptide, a male New Zealand White rabbit was immunized 4 times with 1 mg MAP in 0.5 mL Freund's incomplete adjuvant and 0.5 mL sodium hydrogen phosphate, pH 7.4, at 0, 2, 4, and 14 weeks. After 16 weeks the animals were humanely killed by heart puncture under ketamine (25 mg/kg) and xylazine (5 to 10 mg/kg), which were administered intramuscularly, and their sera were collected. Epitope-specific antibodies were purified by a method to that described previously by coupling 3 mg MAP to agarose gel beads (Affi-Gel 10, Bio-Rad). Similar to the method described for human anti-mHSP65 antibodies,⁹ ELISA plates (Polar Plastic) were coated with the respective MAP (1 μ g/well). After being blocked with 5% dry skim milk (Merck) in PBS, rabbit antisera or purified rabbit antibodies, diluted from 1 in 20 to 1 in 20 480, were incubated for 1 hour at room temperature. The reaction was considered positive when, after incubation with secondary anti-rabbit immunoglobulins (P217, DAKO) and detection with ABTS (Sigma), the optical density at 410 nm exceeded 0.2.

Dot Blots

Recombinant proteins (1 μ g for bacterial HSP60s, 500 ng for hHSP60, 1 μ g ovalbumin) were air-dried on a nitrocellulose membrane (Protran, Schleicher & Schuell) at 37°C for 1 hour. After being blocked with 5% dry skim milk (Merck) in PBS for 30 minutes, the membranes were probed with purified anti-epitope antibodies (10 μ g/mL in 5% dry skim milk) for 1 hour. Reactions were visualized by an enhanced chemiluminescence kit (ECL, Amersham Pharmacia) after incubation with peroxidase-conjugated swine anti-rabbit immunoglobulins (P217, DAKO).

Immunohistochemistry

Four-micron-thick frozen carotid arterial sections of 7 young (8, 9, 16, 19, 25, 26, and 27 years old) clinically healthy donors who had died of accident or suicide were used for immunohistochemistry. After air-drying the slides for 30 to 60 minutes at room temperature, they were fixed in acetone for 10 minutes. Sections were then incubated for 15 minutes with 10% normal human serum (heat-inactivated at 60°C for 30 minutes in Tris-buffered saline [TBS], pH 7.4). Excess serum was blotted off, and the primary antibody-purified, epitope-specific rabbit antibody, diluted 1:10 in TBS, a pooled mixture of all epitope-specific rabbit antibodies at a final dilution for each antibody of 1 in 100 in TBS, or, as a positive control, polyclonal rabbit anti-HSP60, 1 in 100 in TBS (SPA-805, Stressgen), was applied directly without any further washing procedures and incubated for 30 minutes. Slides were rinsed 3 times in TBS, and the secondary antibody, conjugated to alkaline phosphatase (swine anti-rabbit immunoglobulin, DAKO), was incubated for another 30 minutes followed by rinsing in TBS. Visualization was done by adding fast red-naphthol (Sigma), and for better histologic orientation, sections were counterstained with hematoxylin (Merck). Finally, slides were mounted in Kayser's glycerol gelatin (Merck).

Results

Epitope Mapping

The aim of this study was to identify sequence motifs that are shared between human and bacterial HSP60s and that are recognized by purified anti-bacterial HSP60 antibodies from human sera. Titers of the recovered affinity-purified antibodies were high and similar to the titer of the serum pool, whereas no reactivity to ovalbumin was measurable (data not shown). As demonstrated in a previous study by western blot analysis, the purified antibodies not only reacted with the HSP for which they were purified but also recognized their bacterial and mammalian homologues.¹⁸ Anti-mHSP65, anti-chHSP60, and anti-GroEL antibodies were probed on 3 spot membranes, and their reaction with each peptide was densi-

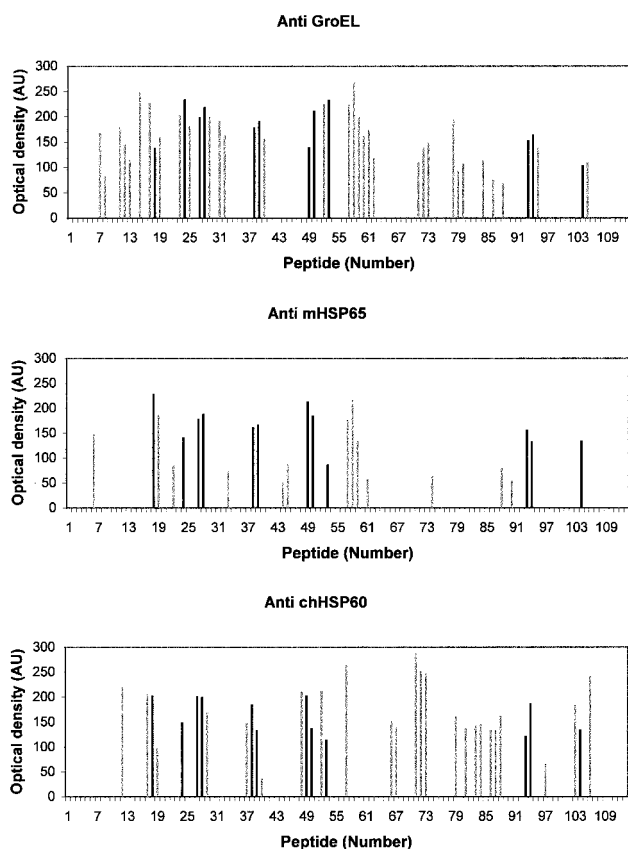


Figure 1. Antibodies to HSP60 from *E coli* (anti-GroEL), *Mycobacterium* (anti-mHSP65), and *Chlamydia* (anti-chHSP60) were purified by affinity chromatography from human sera of patients with atherosclerosis and were probed on membranes with 113 immobilized 13-mer peptides spanning the sequence of human HSP60 with an 8-amino acid overlap. Densitometric quantification revealed 8 cross-reactive epitopes (black bars) that were found to react with all anti-bacterial HSP60 antibodies (peptide Nos. 18, 24, 27/28, 38/39, 49/50, 53, 93/94, and 104).

tometrically quantified. Interestingly, antibodies from both serum pools showed virtually no difference in their epitope pattern. All experiments were performed twice, and only peptides that reacted in all experiments were considered positive. As summarized in Figure 1, the anti-mHSP65 antibodies recognized 25 peptides from hHSP60 (22%), forming 17 linear epitopes. Anti-chHSP60 antibodies showed a reaction with 35 human peptides (31%), which were clustered into 18 epitopes. Forty-five peptides, forming 18 epitopes, were recognized by anti-GroEL antibodies. Eight continuous epitopes on hHSP60 were found to be reactive with all anti-bacterial HSP60 antibodies tested. Four of them consisted of a single 13-mer peptide, and 4 were assembled by 2 adjacent peptides.

Sequence Alignments and Comparative Modeling

The amino acid sequences of hHSP60, mHSP65, GroEL, and chHSP60 were aligned by using CLUSTALW software²⁶ for multiple sequence alignment and the amino acid scoring function for isomorphic replacement by Tudos et al,²⁷ which is suitable for epitope analysis.²⁸ Epitopes 2, 4, and 8 are located in highly conserved regions, with amino acid identity

Epitope 1 (aa 60-72)	hHSP60 LKDKYKNIGAKLV	mHSP65 LEDPYEKIGAEV	GroEL LEDKFNMGAMV	chHSP60 LADKHENMGAMV	Epitope 2 (aa 90-102)	hHSP60 ATVLARSIAKEGF	mHSP60 ATVLAQALVREGL	GroEL ATVLAQAIITEGL	chHSP60 ATVLAEAITYTEGL
	* * . : : * : * : *					***** : : * :			
Epitope 3 (aa 105-122)	hHSP60 ISKGANPVEIRRGVMLAV	mHSP60 VAAGANPLGLKRGIEKAV	GroEL VAAGMNPMDLKRIGDKAV	chHSP60 VTAGANPMDLKRIGDKAV	Epitope 4 (aa 160-177)	hHSP60 ISDAMKKVGRKGVITVKD	mHSP60 IAEAMDKVNGEGVITVEE	GroEL IAEAMDKVNGEGVITVEE	chHSP60 IAEAMEKVGKNGSITVEE
	:: * * : : : * : * *					* : * : * . * * : * * * : *			
Epitope 5 (aa 215-232)	hHSP60 DAYVLLSEKKISSIQSIV	mHSP60 DPYILLVSSKSVTVKDLL	GroEL SPFILLADKKISNIREML	chHSP60 DALVLIYDKKISGKIDFL	Epitope 6 (aa 235-247)	hHSP60 LEIANAHRKPLVI	mHSP60 LEKVIAGKPELLI	GroEL LEAVAKAGKPELLI	chHSP60 LQQAESAQRPELLI
	. . : * : . . * : : : . .					* : . . * : * *			
Epitope 7 (aa 435-452)	hHSP60 QKIGIEIIRKTLKIPAMT	mHSP60 EATGANIVKVALEAPLQK	GroEL QNVGIVKVALRAMEAPLRQ	chHSP60 EQIGARIVLKALSAPLQK	Epitope 8 (aa 490-502)	hHSP60 KGIIDPTKVVTRTA	mHSP60 AGVADPVKVVTRSA	GroEL MGILDPTKVVTRSA	chHSP60 AGILDPAKVVTRSA
	: * . : : : * *					* : * : * . * : *			

Figure 2. Homologue epitope regions as derived by multiple sequence alignment. Identical amino acids are marked with an asterisk, highly conserved positions with a colon, and homologue positions with a period. Amino acid numbering refers to mature hHSP60, ie, without the mitochondrial targeting sequence.

and homology >50% and 80%, respectively (Figure 2). The regions in which the other epitopes are located show average identity and homology. Because there are no experimental structural data available yet for hHSP60, we used a model derived by comparative modeling (SwissModel²⁹), based on Protein Data Bank (<http://www.rcsb.org/pdb/>) entries of 4 bacterial HSP60s (1GRL, 1OEL, 1AON, and 1JON). Owing to sequence homology >70% with the templates, reliability of the model should be very high (root mean square deviation for backbone atoms ≤ 4 Å).³⁰ In the 3-dimensional structure of hHSP60, the epitopes are found to be distributed over the whole protein, and a single immunodominant domain cannot be identified (see Figure 3). Epitopes 1, 2, 3, 7, and 8 are located in the large equatorial domain of HSP60; epitope 4 lies in the intermediate domain; and epitopes 5 and 6 are located in the apical domain. All epitopes are at least partly surface-exposed and accessible to the antibodies.

Peptide Synthesis and Production of Epitope-Specific Antibodies

The 8 identified linear cross-reactive hHSP60 epitopes, recognized by all anti-bacterial HSP antibodies tested, were synthesized by using the concept of MAPs.²⁵ The MAPs obtained had molecular weights between 12 and 16 kDa and were directly immunogenic without coupling to a carrier protein. For each peptide, a rabbit was immunized 4 times (0, 2, 4, and 14 weeks) with 1 mg MAP in Freund's incomplete adjuvant. After 16 weeks, antiserum was collected. All antisera showed high and specific antibody titers against the peptide used as the immunogen, whereas cross-reactivity with the other peptides was not observed (data not shown). Epitope-specific antibodies were purified by affinity chromatography with the respective peptide. The purified anti-

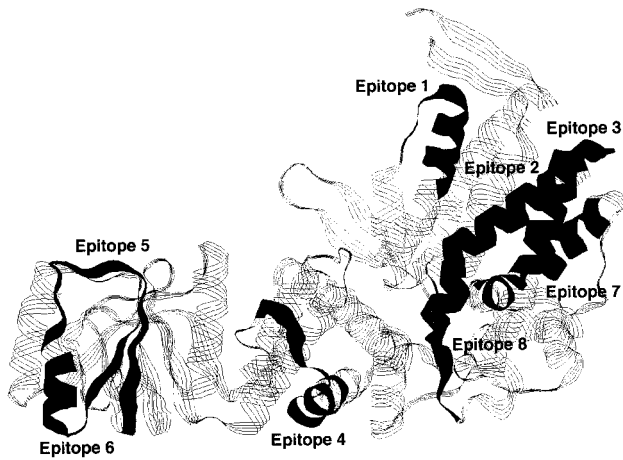


Figure 3. Calculated model of an hHSP60 monomer. The 3-dimensional structure was obtained from comparative modeling by using x-ray structures of 4 bacterial chaperones as templates. The protein consists of 3 domains: apical (left), intermediate (middle), and equatorial (right). Identified cross-reactive continuous epitopes are distributed over the whole protein (depicted in black ribbons). Epitopes 1, 2, 3, 7, and 8 are located in the equatorial domain, epitopes 5 and 6 in the apical domain, and epitope 4 in the intermediate domain.

epitope antibodies, which were raised against peptides derived from hHSP60, reacted not only with hHSP60 but also showed cross-reactivity to nearly all bacterial HSPs by dot blot analysis (Figure 4). The exceptions were anti-epitope 3 antibody, which did not recognize mHSP65, and anti-epitope 7, which did not react with GroEL and chHSP60.

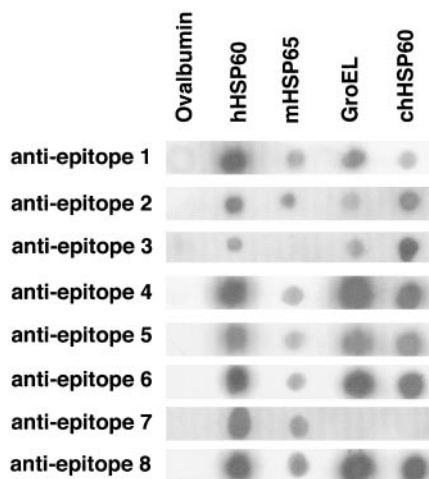


Figure 4. Cross-reactivity of purified antibodies was tested by dot blot analysis. Recombinant HSPs and ovalbumin, serving as a negative control, were spotted on a nitrocellulose membrane and probed with epitope-specific antibodies. Although the antibodies were raised against peptides derived from hHSP60, all showed cross-reactivity with at least some of their bacterial homologues. Anti-epitope 1, 2, 4, 5, 6, and 8 antibodies recognized all microbial HSP60s tested (mHSP65, GroEL, and chHSP60); anti-epitope 3 antibody was reactive only with GroEL and chHSP60, whereas anti-epitope 7 antibody recognized just mHSP65.

Overview of Immunohistochemistry With Purified Anti-Epitope Antibodies

Arterial Section		Atherosclerotic Lesion	Positive Staining Observed
Age, y	Sex		
27	Female	None	None
8	Male	None	Epitope 8
9	Male	None	Epitope 8
19	Male	None	Epitope 8
16	Female	Early	Epitopes 1, 2, 3, 8
25	Female	Early	Epitopes 1, 2, 3, 7, 8
26	Male	Advanced	Epitopes 1, 2, 3, 7, 8
23	Female	Advanced	Epitopes 1, 2, 3, 4, 5, 6, 7, 8

Immunohistochemistry

To determine whether the identified cross-reactive epitopes are present in early atherosclerotic lesions, purified epitope-specific antibodies were tested on arterial sections of 8 young, clinically healthy donors. Identifiable atherosclerotic alterations were already present in the arteries of 4 of these subjects, whereas 2 even showed severe atherosclerotic lesions (Table 1). The antibody against epitope 8 was found to be reactive with 7 of 8 tested arterial sections. In specimens from apparently healthy arteries, this antibody stained cells on the surface of the vessel (Figure 5B). Double-staining experiments with von Willebrand factor identified these cells as endothelial cells (Figure 5C). Antibodies specific for epitopes 1, 2, 3, and 7 were found to be reactive with endothelial cells, subendothelial cells, and foam cells in sections with already detectable atherosclerotic alterations (Figure 5D). Antibodies specific for epitopes 4, 5, and 6 showed significant staining in only 1 arterial section with severe atherosclerotic lesions (for summary, see Table 1). A pooled mixture of all epitope-specific antibodies was applied to the late lesions. Compared with a commercially available rabbit anti-HSP60 antibody, which was produced by immunization with whole protein, a similar staining pattern was observed (<FIG-5>Figure 45 and 5F</FIG>).

Discussion

Autoimmunity to hHSP60, triggered by cross-reactivity of the protective immune response directed against microbial HSP60s, is believed to be involved in many autoimmune diseases, including atherosclerosis.³ In addition to their proven diagnostic potential, a putative causal role for circulating anti-HSP60 antibodies in atherogenesis might involve an autoimmune reaction to endothelial cells that express HSPs as a consequence of different forms of stress, such as local infections, hemodynamic stress, toxins, or biochemically modified LDL.³¹⁻³⁴ In this study, we provide the first evidence that anti-HSP60 antibodies purified from subjects with carotid atherosclerosis recognize distinct, continuous epitopes shared between HSP60 from humans, *Mycobacterium*, *Chlamydia*, and *E coli*. In addition, we demonstrate that some of these epitopes are expressed to different degrees in normal vessels and atherosclerotic lesions.

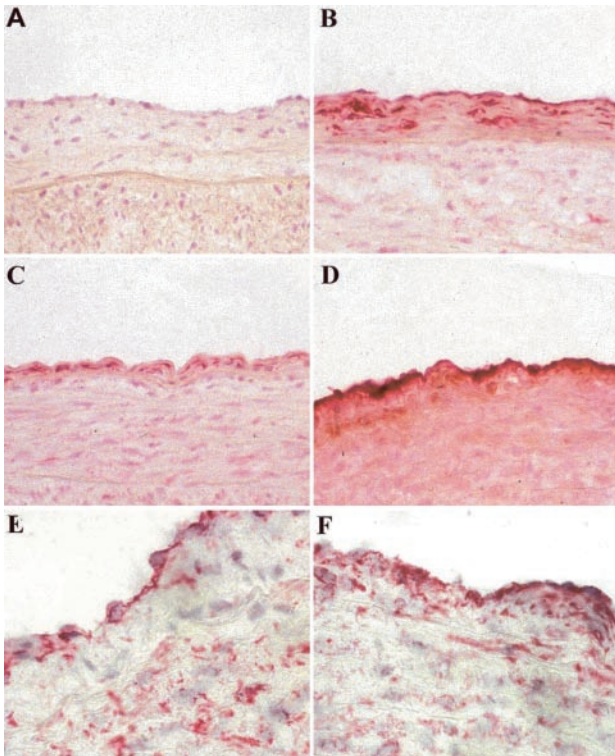


Figure 5. Immunohistochemistry with purified anti-epitope antibodies (vascular lumen always in upper portion of microphotographs): A, Advanced lesion, 200-fold magnification, conjugate control. B, Early lesion, 200-fold magnification, anti-epitope 8 antibody (red). Endothelial and subendothelial cells in the intima are positive. C, Early lesion, 200-fold magnification, anti-epitope 1 antibody (red). Note subendothelial location of positively stained cells. D, Early lesion, 200-fold magnification, double staining with von Willebrand factor as endothelial cell marker (red) and anti-epitope 8 antibody (brown). Note double staining of endothelial cells. Immunohistochemistry of late atherosclerotic lesions: pooled mixture of polyclonal rabbit antibodies against epitopes 1 through 8 compared with commercial rabbit anti-HSP60 antibody: E, Advanced lesion, 600-fold magnification, pooled mixture of 8 purified epitope-specific antibodies. F, Advanced lesion, 600-fold magnification, commercial rabbit anti-HSP60 antibody. Note similar staining patterns in E and F.

Other studies have mapped epitopes on HSP60 homologues from different bacterial species, best recognized by serum antibodies in infectious diseases.^{22,35,36} However, because highly autoreactive B cells and helper T cells would have been eliminated during maturation of the immune system, it is unlikely that the strongest-binding antibodies recognize motifs that are shared between self and non-self molecules of pathogens. The previously reported bacterial HSP60 epitopes seem to be distributed throughout the linear sequence of the molecule, and a general immunodominant region cannot be found. We confirmed these findings on a 3-dimensional structure of the human homologue, where our identified cross-reactive epitopes are also spread over the entire molecule. However, owing to spacial proximity in the modeled tertiary structure of hHSP60, the linear epitopes 2, 3, and 7 and 5 and 6 seem to be assembled into 2 conformational regions (Figure 3).

The nature of the epitopes identified in this study is intrinsically cross-reactive, because we have demonstrated

that antibodies raised in rabbits against human peptides representing our epitopes also recognize bacterial HSP60. Recent publications emphasize the possibility that autoantibodies to hHSP60 might be inborn and at least partly inherited, rather than resulting from stimulation with cross-reacting microbial or biochemically altered autologous epitopes.³⁷ Herein we provide evidence for atherosclerosis-associated epitopes shared between hHSP60 and bacterial HSP60. Because we have been unable to identify humans without anti-bacterial HSP60 antibodies, the question whether bona fide non-cross-reactive anti-hHSP60 autoantibodies contribute to atherogenesis can only be delineated after identification of additional hHSP60-specific epitopes.

Thus, evidence suggests that HSP60 might localize to the surface of eukaryotic cells,^{5,6,38} although less is known about its function and structure on the cell surface. Recent investigations provide evidence for the presence of an HSP60 receptor,^{39,40} indicating that the HSP found on the cell surface might come not only from the cell itself but also originate from external sources. We have previously reported that the anti-hHSP60 monoclonal antibody II-13 is cytotoxic for stressed endothelial cells, whereas another monoclonal antibody, ML-30, which recognizes a different epitope, is not.⁵ Thus, only distinct epitopes are accessible for antibodies, suggesting that the surface orientation of HSP60 is important. It also cannot be excluded that just single domains of hHSP60 are present on the outer surface of the cells.

It is known that in Western countries >50% of children aged 10 to 12 years have already ongoing atherosclerotic alterations in their coronary arteries.⁴¹ Because most of them lack any known classic risk factor, we have studied the arteries of children or young adults by immunohistochemistry. In manifest atherosclerotic lesions, all 8 epitopes were recognized, and intimal cells and foam cells stained particularly strongly (Figure 5A–5D). Interestingly, in very early lesions, a different situation was found: Here, only antibodies directed against epitopes located in the equatorial domain (1, 2, 3, 7, and 8) were reactive, suggesting a defined orientation of hHSP60 on the cell surface that allows antibodies to react with only this part of the molecule. The antibody directed against epitope 8 was especially very reactive in apparently healthy arterial specimens of children, particularly at branching arterial regions, which are subjected to turbulent blood flow stress. Thus, antibodies reactive to epitope 8 might be involved in the development of early inflammatory disorders in prestressed regions of the arterial wall.

However, a pooled mixture of the epitope-specific antibodies showed immunohistochemical recognition patterns similar to a commercially available anti-HSP60 antibody (Figure 5E and 5F). In the case of late atherosclerotic lesions, inflammatory processes, including infiltration of mononuclear cells and the release of locally produced cytokines, might be strong HSP60 inducers, which make most of the epitopes accessible to antibodies. From these findings, we conclude that our cross-reactive HSP60 epitopes identified in sera from subjects with incipient atherosclerosis might provide further insights into the mechanisms of early atherogenesis and serve as promising targets for future diagnostic and therapeutic approaches.

Acknowledgments

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References

- Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999;340:115–126.
- Karlin S, Brocchieri L. Heat shock protein 60 sequence comparisons: duplications, lateral transfer, and mitochondrial evolution. *Proc Natl Acad Sci U S A*. 2000;97:11348–11353.
- Zugel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin Microbiol Rev*. 1999;12:19–39.
- Wand-Wurtenberger A, Schoel B, Ivanyi J, Kaufmann SH. Surface expression by mononuclear phagocytes of an epitope shared with mycobacterial heat shock protein 60. *Eur J Immunol*. 1991;21:1089–1092.
- Xu Q, Schett G, Seitz CS, Hu Y, Gupta RS, Wick G. Surface staining and cytotoxic activity of heat-shock protein 60 antibody in stressed aortic endothelial cells. *Circ Res*. 1994;75:1078–1085.
- Soltys BJ, Gupta RS. Cell surface localization of the 60 kDa heat shock chaperonin protein (hsp60) in mammalian cells. *Cell Biol Int*. 1997;21:315–320.
- Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? *Lancet*. 1997;350:430–436.
- Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E, Oberhollenzer F, Muggeo M, Xu Q, Wick G, Poewe W, Willeit J. Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. *Circulation*. 2001;103:1064–1070.
- Xu Q, Willeit J, Marosi M, Kleindienst R, Oberhollenzer F, Kiechl S, Stulnig T, Luef G, Wick G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. *Lancet*. 1993;341:255–259.
- Xu Q, Kiechl S, Mayr M, Metzler B, Egger G, Oberhollenzer F, Willeit J, Wick G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis: clinical significance determined in a follow-up study. *Circulation*. 1999;100:1169–1174.
- Birmie DH, Holme ER, McKay IC, Hood S, McColl KE, Hillis WS. Association between antibodies to heat shock protein 65 and coronary atherosclerosis: possible mechanism of action of *Helicobacter pylori* and other bacterial infections in increasing cardiovascular risk. *Eur Heart J*. 1998;19:387–394.
- Frostegard J, Lemne C, Andersson B, van der Zee R, Kiessling R, de Faire U. Association of serum antibodies to heat-shock protein 65 with borderline hypertension. *Hypertension*. 1997;29:40–44.
- Burian K, Kis Z, Virok D, Endresz V, Prohaszka Z, Duba J, Berencsi K, Boda K, Horvath L, Romics L, Fust G, Gonczol E. Independent and joint effects of antibodies to human heat-shock protein 60 and *Chlamydia pneumoniae* infection in the development of coronary atherosclerosis. *Circulation*. 2001;103:1503–1508.
- Zhu J, Qiyumi AA, Rott D, Csako G, Wu H, Halcox J, Epstein SE. Antibodies to human heat-shock protein 60 are associated with the presence and severity of coronary artery disease: evidence for an autoimmune component of atherogenesis. *Circulation*. 2001;103:1071–1075.
- Huittinen T, Leinonen M, Tenkanen L, Manttari M, Virkkunen H, Pitkanen T, Wahlstrom E, Palosuo T, Manninen V, Saikku P. Autoimmunity to human heat shock protein 60, *Chlamydia pneumoniae* infection, and inflammation in predicting coronary risk. *Arterioscler Thromb Vasc Biol*. 2002;22:431–437.
- Schett G, Xu Q, Amberger A, Van der Zee R, Recheis H, Willeit J, Wick G. Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. *J Clin Invest*. 1995;96:2569–2577.
- Schett G, Metzler B, Mayr M, Amberger A, Niederwieser D, Gupta RS, Mizzen L, Xu Q, Wick G. Macrophage-lysis mediated by autoantibodies to heat shock protein 65/60. *Atherosclerosis*. 1997;128:27–38.
- Mayr M, Metzler B, Kiechl S, Willeit J, Schett G, Xu Q, Wick G. Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of *Escherichia coli* and *Chlamydia pneumoniae*: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. *Circulation*. 1999;99:1560–1566.
- Xu Q, Dietrich H, Steiner HJ, Gown B, Schoel B, Mikuz G, Kaufmann SH, Wick G. Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65. *Arterioscler Thromb*. 1992;12:789–799.
- Afek A, George J, Gilburd B, Rauova L, Goldberg I, Kopolovic J, Harats D, Shoenfeld Y. Immunization of low-density lipoprotein receptor deficient (LDL-RD) mice with heat shock protein 65 (HSP-65) promotes early atherosclerosis. *J Autoimmun*. 2000;14:115–121.
- Mosorin M, Surcel HM, Laurila A, Lehtinen M, Karttunen R, Juvonen J, Paaonon J, Morrison RP, Saikku P, Juvonen T. Detection of *Chlamydia pneumoniae*-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler Thromb Vasc Biol*. 2000;20:1061–1067.
- Metzler B, Schett G, Kleindienst R, van der Zee R, Ottenhoff T, Hajeer A, Bernstein R, Xu Q, Wick G. Epitope specificity of anti-heat shock protein 65/60 serum antibodies in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 1997;17:536–541.
- Wick G, Perschinka H, Xu Q. Autoimmunity and atherosclerosis. *Am Heart J*. 1999;138:S444–S449.
- Willeit J, Kiechl S. Prevalence and risk factors of asymptomatic extracranial carotid artery atherosclerosis: a population-based study. *Arterioscler Thromb*. 1993;13:661–668.
- Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci U S A*. 1988;85:5409–5413.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994;22:4673–4680.
- Tudos E, Cserzo M, Simon I. Predicting isomorphous residue replacements for protein design. *Int J Pept Protein Res*. 1990;36:236–239.
- Davies JM, Scaely M, Cai YP, Whisstock J, Mackay IR, Rowley MJ. Multiple alignment and sorting of peptides derived from phage-displayed random peptide libraries with polyclonal sera allows discrimination of relevant phagotopes. *Mol Immunol*. 1999;36:659–667.
- Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 1997;18:2714–2723.
- Sanchez R, Pieper U, Melo F, Eswar N, Marti-Renom MA, Madhusudhan MS, Mirkovic N, Sali A. Protein structure modeling for structural genomics. *Nat Struct Biol*. 2000;7(suppl):986–990.
- Seitz CS, Kleindienst R, Xu Q, Wick G. Coexpression of heat-shock protein 60 and intercellular-adhesion molecule-1 is related to increased adhesion of monocytes and T cells to aortic endothelium of rats in response to endotoxin. *Lab Invest*. 1996;74:241–252.
- Frostegard J, Kjellman B, Gidlund M, Andersson B, Jindal S, Kiessling R. Induction of heat shock protein in monocytic cells by oxidized low density lipoprotein. *Atherosclerosis*. 1996;121:93–103.
- Amberger A, Maczek C, Jurgens G, Michaelis D, Schett G, Trieb K, Eberl T, Jindal S, Xu Q, Wick G. Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. *Cell Stress Chaperones*. 1997;2:94–103.
- Hochleitner BW, Hochleitner EO, Obrist P, Eberl T, Amberger A, Xu Q, Margreiter R, Wick G. Fluid shear stress induces heat shock protein 60 expression in endothelial cells in vitro and in vivo. *Arterioscler Thromb Vasc Biol*. 2000;20:617–623.
- Yi Y, Zhong G, Brunham RC. Continuous B-cell epitopes in *Chlamydia trachomatis* heat shock protein 60. *Infect Immun*. 1993;61:1117–1120.
- Maeda H, Miyamoto M, Kokeguchi S, Kono T, Nishimura F, Takashiba S, Murayama Y. Epitope mapping of heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*. *FEMS Immunol Med Microbiol*. 2000;28:219–224.
- Prohaszka Z, Duba J, Horvath L, Csaszar A, Karadi I, Szebeni A, Singh M, Fekete B, Romics L, Fust G. Comparative study on antibodies to human and bacterial 60 kDa heat shock proteins in a large cohort of patients with coronary heart disease and healthy subjects. *Eur J Clin Invest*. 2001;31:285–292.
- Soltys BJ, Gupta RS. Immunoelectron microscopic localization of the 60-kDa heat shock chaperonin protein (Hsp60) in mammalian cells. *Exp Cell Res*. 1996;222:16–27.
- Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J Immunol*. 2000;164:13–17.
- Ohashi K, Burkart V, Floh S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol*. 2000;164:558–561.
- PDAY Study Group. Natural history of aortic and coronary atherosclerotic lesions in youth: findings from the PDAY Study: Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. *Arterioscler Thromb*. 1993;13:1291–1298.