Immunity to heat shock protein 65—an additional determinant in intimal thickening

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Abstract

Inflammation occurring consequent to vessel injury is thought to play an important role in atherosclerosis and restenosis. Autoimmunity to HSP65 has been shown to accelerate early atherogenesis in rabbits and mice, whereas in humans epidemiological data support this contention. In the current study, we explored the possibility of HSP65 influencing the extent of neointimal growth in the rat carotid injury model. Rats were either immunized with recombinant mycobacterial HSP65, heat killed preparation of Mycobacterium tuberculosis (MT), or with PBS, all emulsified in incomplete Freund’s adjuvant. Animals were boosted with a similar protocol 3 weeks following the primary immunization and 2 weeks later carotid injury was applied in all animals by balloon inflation. Upon sacrifice 2 weeks later, sera were obtained for measurement of anti-HSP65 antibodies by ELISA, splenocytes were assessed for proliferative response to in vitro priming with HSP65, and carotid arteries were removed for evaluation of neointimal growth. Rats immunized with HSP65 exhibited a brisk and sustained humoral immune response to HSP65, and cellular immunity was also evident by thymidine uptake to splenocytes primed with the respective protein. Neointimal/medial ratio was significantly increased in HSP65 immunized rats, in comparison with MT injected and control animals. In conclusion, immunity to HSP65 can play a role in accelerating restenosis following arterial injury. These results should be further investigated in humans as they may provide a possible link between infections and restenosis/accelerated arteriosclerosis.

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1. Introduction

Heat shock proteins are a group of proteins that are thought to evolve to confer protection against various forms of cellular stress [1,2]. However, the protective properties afforded by HSPs can turn and inflict forms of pathological autoimmunity [3,4]. This notion has been proven in experimental models where footprints of autoimmune reactions to HSP65 have been associated with arthritis [5] and diabetes [6]. The relevance of these findings to humans is principally derived from the concept of antigenic mimicry. According to this paradigm, infectious agents bearing bacterial HSP65 can induce a respective immune response that cross reacts with self expressed (mammalian) HSP to result in a local ‘autoimmune’ reaction [4].

The proof of concept for the presence of antigenic mimicry has also been thoroughly investigated experimentally in atherosclerosis (reviewed in [7,8]). Normocholesterolemic rabbits immunized against mycobacterial HSP65 developed arteriosclerotic plaques irrespective of their cholesterol levels [9]. Host HSP65 was subsequently traced in the plaques of rabbits [10], and in the vessel wall of injured rat carotid arteries [11], providing the ligand to which HSP65 antibodies/T cells

Abbreviations: ELISA, enzyme linked immunosorbent assay; HSP, heat shock protein; IFA, incomplete Freund’s adjuvant; MT, Mycobacterium tuberculosis.

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would be attached. We confirmed the results in rabbits, employing naive and transgenic LDL receptor deficient mice, demonstrating that immunization with HSP65 led to enhancement of early atherosclerotic lesions [12,13]. Additionally, bacterial HSP65 has been localized to human atheroma, regulating TNF-α and matrix metalloproteinase production [14], as well as activating smooth muscle cells and macrophages [15]. Data obtained from humans support the results obtained experimentally by attributing an important role for anti-HSP65 antibodies in association with atherosclerosis and by predicting clinical outcome [16–18].

Restenosis is a process in which prior vascular injury results in lumen obliteration by cell migration and proliferation with consequent formation of a neointima [19]. The restenotic process bears several similarities to atherosclerosis. Both processes are believed to follow stress to the vessel wall and are associated with cell migration and proliferation. Additionally, recent evidence attributes an important role for inflammation in the evolution of both atherosclerotic and restenotic lesions [19–21]. Among the results supporting this contention are the studies linking infections to atherosclerosis/restenosis (reviewed in [22,23]).

In the current paper we tested the hypothesis that induction of HSP65 autoimmunity would result in enhancement of intimal thickening in a rat model of arterial injury.

2. Materials and methods

2.1. Animals

Wistar rats 8 weeks old were obtained from the Tel Aviv University and bred at the local animal house (Tel Aviv Medical Center). The animals were housed in plastic cages with controlled 12-h light: 12-h dark cycles and allowed access to food and water ad-libitum.

2.2. Experimental design

A preliminary study was carried out to determine whether differences in neointimal thickening are induced by immunization with a control antigen. Five rats were immunized with 100 μg of bovine serum albumin (BSA) emulsified in incomplete Freund’s adjuvant (IFA), and boosted with a similar protocol 3 weeks later. Control rats (n = 6) were immunized with PBS in IFA, and boosted with a similar protocol 3 weeks later. The third group consisted of non-treated, injured rats.

In the principal experiment, rats were divided into three groups according to the immunizing agent:

Group A: Rats (n = 8) immunized with 100 μg of mycobacterial HSP65 (kindly provided by Dr Singh, GBF) emulsified in IFA and boosted with a similar protocol, 3 weeks later.

Group B: Rats (n = 7) immunized with 100 μg of heat killed preparation of Mycobacterium tuberculosis (MT), emulsified in IFA and boosted with a similar protocol, 3 weeks later (similar to the preparation described in [12]).

Group C: Rats (n = 7) immunized with PBS in IFA and boosted with a similar protocol, 3 weeks later.

Two weeks after boost, the animals were bled and carotid injury was induced. Two weeks following induction of vascular injury, the animals were sacrificed, the carotid arteries obtained for morphometric analysis, spleen cells removed for proliferation assays and sera obtained for evaluation of antibody levels.

2.3. Rat carotid injury model

Rats were anesthetized by intraperitoneal injection of ketamin (80 mg/kg) and xylazine (5 mg/kg). Endothelial denudation and vascular injury was performed in the left common carotid artery, as described [24]. Briefly, a balloon catheter (2F Fogarty) was advanced though the external carotid into the aorta; the balloon was inflated with sufficient water to distend the common carotid artery and then pulled back to the external carotid. This procedure was repeated three times, and then the catheter was removed. After 14 days, the animals were sacrificed by a lethal dose of phenobarbital sodium and booth the right and left carotid arteries were removed and fixed in 4% paraformaldehyde until embedding in paraffin.

2.3.1. Histological assessment of intimal lesions

Serial cross sections (5 μm thick) were used throughout the entire length of the carotid artery for histological analysis (average of five per animal). All samples were routinely stained with hematoxylin and eosin or and masson-trichrome stains (five sections for each).

To evaluate inflammatory cell numbers in the vessel wall, sections were stained with rabbit anti iNOS2 antibody, (Santa Cruz Biotechnology). Positively stained cells were counted and averaged from sections of all animals from each group.

2.3.2. Quantification of intimal lesions in sections of carotid arteries

Five equally spaced cross sections were used in all rats to quantify intimal lesions. Using image analysis software (Professor I. Hammel, Department of Pathology, Tel Aviv University), total cross-sectional medial area was measured between the external and internal elastic laminae. Total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.
2.3.3. Cholesterol level determinations

Total plasma cholesterol levels were determined by using an automated enzymatic technique (Boehringer Mannheim, Germany).

2.4. Lymphocyte proliferation assays

Splenocytes were collected from four HSP-65, MT, and IFA immunized rats upon sacrifice. The assays were performed as previously described [25] with minor modifications. Briefly, $1 \times 10^6$ cells/ml were incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence or absence of 10 μg/ml HSP-65. Proliferation was measured by the incorporation of $[^3]H$ thymidine into DNA during the final 12 h of incubation. The results were computed as stimulation index (SI): the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen. Standard deviation was always $<10\%$ of the mean cpm.

2.5. Detection of anti-HSP65 antibodies by ELISA

Recombinant HSP65 (1 μg/ml) in phosphate buffered saline (PBS, pH 7.2) was coated onto flat bottom 96-well ELISA plates (Nunc Maxisorp, Denmark) by overnight incubation. After washings with 0.02% PBS Tween and blocking with 1% BSA in PBS, sera (in a dilution of 1:100) were added in different concentrations and incubated for 1 h at room temperature. Alkaline phosphatase conjugated goat anti-rat IgG (DAKO Ltd, High Wycombe, UK) was added and incubated for 1 h at room temperature followed by four washings with PBS/Tween. After extensive washing, 1 mg/ml p-nitrophenyl-phosphate (Sigma) in 50 mM carbonate buffer containing 1 mM MgCl$_2$ pH 9.8 was added as a substrate. The reaction was stopped after 30 min by adding 1 M of NaOH. The optical density (OD) was read at a 405 nm wavelength in a Titertek ELISA reader.

![Fig. 1. Cellular immunity to HSP65](image)

2.6. Statistical analysis

Comparisons among the three groups were made using a one way ANOVA. $P < 0.05$ was considered statistically significant and results are expressed as mean ± S.E.M.

3. Results

A preliminary study set out to evaluate the effect of injection of a control antigen (BSA) and PBS (in IFA) as compared with no treatment, on intimal thickening. It demonstrated that intimal/medial ratio did not differ between the three groups (data not shown). The principal experiments were thus performed with PBS in IFA as the control-immunizing agent.

Total cholesterol levels at the end of the experiment did not differ significantly between rats immunized with HSP65 (2.7 ± 0.4 mmol/l) MT (2.5 ± 0.5 mmol/l) or PBS (2.9 ± 0.4 mmol/l).

Immunization with recombinant mycobacterial HSP65 resulted in a pronounced splenocyte proliferation to HSP65 (SI of 6.1 ± 1.1), compared with IFA immunized rats (SI of 1.1 ± 0.2; $P < 0.001$). Although to a lesser extent than HSP65, immunization with MT was also effective in inducing a proliferative response to HSP65 (SI of 3.8 ± 0.8; $P < 0.01$ in comparison with IFA immunized) (Fig. 1).

IgG anti-HSP65 antibody levels, which were induced following immunization with HSP65, began rising at 10 days following the primary immunization and peaked 1 week after boost injection and remained at similar levels until sacrifice. Antibody levels at the time of sacrifice are shown in Fig. 2. MT immunization also resulted in elevated levels albeit significantly lower levels following immunization with HSP65 (Fig. 2).

Rats immunized with HSP65 had increased neointimal thickness in comparison with IFA or MT immunized animals (data not shown). No differences were evident between groups with respect to medial size.

The neointimal/medial ratio increased in HSP65 immunized animals (1.66 ± 0.14) when compared with IFA (0.84 ± 0.24; $P < 0.01$), or MT immunized (0.88 ± 0.22; $P < 0.01$) rats (Fig. 3).

Immunization with HSP65 rich MT did not significantly influence restenosis. This was evidenced by similar intimal size and intimal/medial ratio in comparison with IFA immunized rats.

No differences were apparent in percentage of inflammatory cells (iNOS positive) in the vessel walls between the three groups (mean of 12.3 ± 2.9, 10.8 ± 3.1 and 11.3 ± 2.0% for groups A, B and C, respectively).
very high levels of anti-HSP65 levels [12]. Cellular immunity to HSP65 was also pronounced in the HSP65 immunized rats, which we confirmed by thymidine incorporation assays performed on HSP65-primed splenocytes. Immunization with MT resulted in an increased splenocyte proliferation to HSP65 but to a lesser extent than immunization with HSP65. The lack of a pronounced immune response to HSP65 in MT immunized rats is probably due to the relatively minor amounts of HSP65 present in MT, in comparison to the doses injected when using the pure recombinant protein. An additional explanation may be the more efficient presentation of the protein obtained following emulsification of the HSP65 with the adjuvant (IFA).

We found that animals that exhibited an enhanced autoimmune response to HSP65 had increased neointimal size and neointimal/medial ratio when compared with control animals. HSP65 is a protein found abundantly in infectious agents, classic examples of which include MT and *Chlamydia pneumonia*. Recent data indicate that infectious agents may be involved in promoting restenosis in humans [23], a finding that may pave the way for new strategies for the prevention of this common disorder. However, despite these findings, no mechanism has yet been found to explain the effect of infections on intimal thickening.

Some suggest that autoimmunity to HSP65 plays a role in the pathogenesis of experimental autoimmune diseases [3–7]. It was consequently proposed that molecular mimicry between infectious and host HSP65 may result in the direction of host immunity towards its own tissues culminating, in autoimmune target-organ damage and atherosclerosis [8]. Supporting this idea is the finding that antibodies to HSP65 are capable of inducing endothelial cell cytotoxicity [27,28] and macrophage lysis [29].

Although it is recognized that intimal thickening is basically a proliferative disorder, where migration of cells forming the neointima is a key factor inflammation has been demonstrated to play an active role in its pathogenesis [30,31]. We recently reinforced this contention by demonstrating that modulating the immune system of mice with intravenous gammaglobulins resulted in reduced intimal thickening [32]. Several factors are thought to influence the extent of the restenotic process, amongst which endothelialization of the denuded area is considered of prime importance. Thus, generation of HSP65 antibodies that promote endothelial toxicity [27,28] are expected to delay endothelialization, thus possibly promoting intimal growth. However, additional mechanisms could be operable such as generation of proinflammatory cytokines by HSP65 reactive lymphocytes.

Restenosis in humans following percutaneous transluminal coronary angioplasty (PTCA) is the result of two major processes: intimal hyperplasia and negative
remodeling [19]. The stent era has reduced the restenosis rate by abolishing the negative remodeling mechanism, but significant numbers of patients still develop narrowed vessels induced by intimal hyperplasia. Our future plans are to investigate the role of HSP65 immunity in experimental restenosis models following stent implantation. In this context, it is important to emphasize the limitations of the study in extrapolating the conclusions to human restenosis. It appears that the rat carotid injury model does not imitate restenosis in humans, as the induced damage is performed differently and the time course and kinetics of intimal thickening are also different. Accordingly, several pharmacological strategies that had shown success in the rat model did not appear successful in humans [33,34]. Thus, the results of our study should be viewed with caution and should be further supported by additional studies in humans.

In conclusion, we have found that induction of mycobacterial HSP65 immunity results in increased neointimal growth, providing a possible mechanism by which infectious agents may promote restenosis in humans. Additional studies should be carried out to confirm the validity of these findings in humans.

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References


