PREFACE

Atherosclerosis is a prolonged process that starts in the second decade of life, progresses slowly and the clinical manifestations appear in the 4–5th decade onwards. The early lesions, i.e., fatty streaks, may be seen already during fetal development and regress soon after birth [1], but reappear in early adolescence and may progress into more complex lesions, that take many years to regress [2]. Evidence based on large studies provided proof that hypercholesterolemia is a leading risk factor for atherogenesis [3]. In addition, other risk factors, such as hypertension, smoking, diabetes, low HDL, homocysteinemia were identified.

Within the last two decades it was recognized that risk factors interact to promote development of coronary artery disease (CAD) [3]. Thus, smoking as a single risk factor, manifests clinically as a respiratory disease, but in conjunction with high LDL cholesterol levels enhances CAD very markedly [4]. The same is true for hypertension and diabetes, that in presence of hypercholesterolemia will increase the risk for CAD several-fold. It has been generally accepted that the known risk factors account for only 50% of patients with CAD [5]. One plausible explanation for underestimation of the contribution of known risk factors to mortality and morbidity of CAD could be that the definition of "normality" requires more rigorous re-evaluation. Three decades ago, 300 mg/dL of plasma cholesterol, 180 mg/dL of LDL cholesterol, blood pressure of 160/95 mm Hg, were considered to be within *upper normal* limits. Information from large-scale intervention studies for secondary prevention of CAD permitted to redefine these limits. Thus the upper normal limit for LDL cholesterol has been continuously decreasing to <100 mg/dL, and recently even <80 mg/dL is considered as a therapeutic goal. The latter values start to approach those found in free living non-human primates, who also develop atherosclerosis when exposed to "western type" diets.

Since the formulation by R.Ross of the "response to injury hypothesis of atherosclerosis" [6–9], it became accepted that this process is an outcome of a chronic inflammatory reaction in the artery. This book deals with knowledge accrued in the past decade concerning the role of immunity in the initiation and perpetuation of atherosclerosis [10–13]. The participation of monocyte derived macrophages and lymphocytes, the latter mostly activated T-cells [14] pointed to the involvement of the immune system in this inflammatory reaction. There is convincing evidence that LDL, once modified by macrophages within the arterial wall, can be responsible for T-cell activation [15]. Circulating antibodies to oxidized LDL have been demonstrated in animals and humans [15]. Immune activation was invoked also for other antigens, such as heat shock proteins [16, 17]. The above mentioned antigens were found to be present in atherosclerotic lesions [15, 18]. In IL-4 knockout mice fewer atherosclerotic lesions after heat shock protein injection were seen, indicating an important role of this cytokine in the response to heat shock protein [19]. Involvement of CD40 and CD40 ligand in atherogenesis (mediators of immune responses) is supported by their expression in most cellular elements of the atheroma [20]. In LDL receptor deficient mice, injection of anti-CD40L antibodies reduced atherosclerosis markedly [20]. In addition, various infections of microbial (chlamydia pneumoniae) or viral (adenoviruses and herpesviridae) origin [21, 22] have been proposed as candidates for vascular injury which may culminate in atherogenesis. Among them herpesviruses were shown to activate the coagulation system and accelerate progression of atherosclerosis [23].

The immune approach to atherosclerosis led also to the use of immunization against oxidized LDL. Thus injection of homologous malonyl dialdehyde modified LDL (MDA-LDL) to WHHL rabbits resulted in reduction of atherosclerosis [24]. Similar results were obtained also in apoE deficient mice hyperimmunized with homologous MDA-LDL [25].

On the other hand, immunization to heat shock proteins of mice fed an atherogenic diet resulted in enhancement of fatty streak formation [26]. In analogy, when LDL receptor deficient [27] and apoE deficient mice were immunized to β-2-glycoprotein I (a target of autoimmune anticardiolipin antibodies) they responded with an accelerated development of atherosclerosis [28]. This antigen was found also in human atherosclerotic lesions co-localizing with CD4-lymphocytes [29]. When primed lymphocytes from β2GPI immunized mice were injected into LDL-receptor deficient mice, atherosclerotic lesions were enhanced [30].

Future research that will be designed to test the relative importance of the immune hypothesis in atherogenesis will determine the approach to be taken in CAD prevention and treatment. In the meantime, introduction of a primary prevention program that encompasses all known risk factors for CAD, throughout the school curriculum, seems to us a logical approach, and with the escalating costs of clinical care of CAD becomes an inescapable conclusion.

Olga Stein and Yechezkiel Stein
Jerusalem, 16th July 2000

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Atherosclerosis and Autoimmunity
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Introduction: Autoimmunity as an Additional 'Risk Factor' for Atherosclerosis

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This book deals with autoimmune aspects of atherosclerosis.

Atherosclerosis is the most prevalent disease responsible for death from myocardial infarction, cerebrovascular events and renal failure. Hardening of the arteries and narrowing of their lumen results from the deposition of macrophages laden with lipids, mainly oxidized LDL and formation of the atherosclerotic plaque. For years the traditional risk factors such as high cholesterol levels hypertension, diabetes mellitus, smoking, family history and others were believed to be the major factors playing in the pathogenicity of atherosclerosis. In the last decade it has been realized that atherosclerosis, an inflammatory process may have infectious and *autoimmune components*. Correlation between many inflammatory markers such as CRP, adhesion molecules, cytokines and others with the incidence and severity of the disease were reported. Bacteria, such as Chlamidya, and viruses, such as EBV and CMV, were implicated in the pathogenesis of the disease and several reports have shown beneficial effects of an antibiotic treatments. Last but not least, autoantigens and autoantibodies seem to be involved in the induction of atherosclerosis. This main autoantigens being heat shock proteins, oxLDL and B2GPI. The later findings were also followed by successful trials to immunomodulate the disease.

Atherosclerosis is a process that involves lipid accumulation in the walls of arteries leading to a compromised flow to nearly any target organ. The final common pathways of advanced atherosclerosis is ischemia of the heart (myocardial infarction), brain (cerebrovascular events) and to the legs (claudication and necrosis).

The modern view of atherosclerosis is that of a chronic inflammatory disorder. It is based on the realization that immune-competent cells are abundant in the vicinity of the plaque starting from its initial stages (i.e. fatty streak formation). Thus, activated T lymphocytes macrophages (the major components of the evolving plaque) and major histocompatibility complex (MHC) bearing cells (i.e. endothelial and smooth muscle cells) constitute, invariable relations in the atherosclerotic plaque.

A recent 'extension' of the inflammatory view of atherosclerosis has been provided in recent years by several laboratories including ours. Thus, it has been speculated that autoimmunity to various autoantigens can act to influence the fate of the advancing lesion.

To demonstrate that a disease has an autoimmune component, we have to show that upon immunization of a naive animal with the implicated autoantigen, the disease can be modified. Specifically, it was shown that autoimmunity to heat shock protein 60 plays an initiatory role in the development of atherosclerosis in experimental animals and in humans. Similar results were achieved following immunization with another compound called ß2GPI. The later is also the autoantigen in SLE patients and in subjects having the antiphospholipid syndrome.

The third candidate autoantigen the oxidized LDL known to be the noxious agent in atherosclerosis was found to be protective in the disease upon active immunization.

The induced disease, like the natural one was characterized by many autoimmune features such as deposition of autoantigens, autoantibodies and involvement of the immune cells the lymphocytes.

The ultimate evidence that indeed atherosclerosis has an autoimmune component is based on our ability to transfer the disease from an induced mice to a naive mice just by transferring the lymphocytes.

It is not surprising that if atherosclerosis has an autoimmune component, that it can be treated by immunomodulation. Indeed, favorable results were achieved with high dose intravenous immunoglobulin (IVIG) which is employed also in other autoimmune diseases such as SLE, polydermatomyositis and by depletion of lymphocytes. Anti CD40/Ligand antibodies treatment and other molecules were also been used successfully by immunomodulating the disease.

Thus atherosclerosis is an additional disease found to have an autoimmune pathogenesis and treatment.

In this book world known experts gathered following a meeting that took place on March 2001 in Geneva, Switzerland. It summarized the diverse aspects of the interrelationship between the immune system and atherosclerosis.

Atherosclerosis and Autoimmunity
Y. Shoenfeld, D. Harats and G. Wick, editors

The Autoimmune Pathogenesis of Atherosclerosis – An Evolutionary-Darwinian Concept

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1. INTRODUCTION

During the last decade, the main classical theories of atherogenesis, i.e. the "response-to-injury" and the "altered lipoprotein" hypotheses and their modifications have been supplemented by the concept that immunologic-inflammatory processes play a major role at various stages of this disease. Interestingly, these ideas about the formation of atherosclerotic lesions have already been discussed since the 19th century. For a long time it has, however, not been clear if the observed inflammatory phenomena in the arterial wall were of a primary or secondary nature. The indications for a participation of immunologic-inflammatory processes in the development of atherosclerotic lesions included the deposition of immunoglobulins and co-distributed complement components in the arterial wall, the participation of lymphoid cells in addition to macrophages in the intimal mononuclear infiltrate, and the occurrence of pro-inflammatory cytokines, chemokines and growth factors and their receptors. In addition, serum markers of inflammation, such as acute phase proteins and indicators for activation of the immune system, e.g. neopterin, were found to be correlated with the occurrence of atherosclerotic lesions. Most importantly, infections have been discussed as possible atherosclerosis-associated or even causally relevant phenomena. In this respect, viral and bacterial infections were discussed, the former including herpes viruses and cytomegaloviruses (CMV), the latter including *Chlamydiae, Helicobacter pylori* and others. Finally, *bona fide* autoimmune reactions against biochemically altered autoantigens, e.g. oxidized low density lipoproteins (oxLDL), have also been considered as possible causes for or phenomena connected with the development of atherosclerosis.

Based on these data and on our own long standing interest in experimentally induced and spontaneously occurring animal models for autoimmune diseases and their respective human counterparts as well as our previous work on the role of the lipid metabolism for lymphocyte reactivity with special emphasis on age-related changes of the immune response, we decided more than a decade ago to investigate the question if humoral and/or cellular immune reactions play an *initiating* role in the development of atherosclerosis. In case that these endeavours were successful, we, of course, finally aimed at identifying the antigen(s) that is/are possibly involved in this process. Our work, that has in the meantime been supported by data from many other laboratories, has shown that the antigen in question is a certain stress protein, heat shock protein 60 (HSP 60). As will be detailed below, HSP 60 is expressed by vascular endothelial cells upon being subjected to various forms of stress, mainly including those that are already known as classical risk factors for atherogenesis.

In this contribution, we will take atherosclerosis as a paradigmatic age-related disease that can be taken as a price that we pay later in life for genetic traits that are of benefit in younger years, i.e. up to the time of reproduction. As we will see, in the case of atherosclerosis we pay for our potential to raise protective immunity against microbial (viral, bacterial, parasitic) HSP 60 that allows for survival until

the time of reproduction, but that may be detrimental later on under circumstances that have not been subject to evolutionary pressure since reaching an old age and living beyond the fertility period of an indivudual was obviously not "forseen" by nature.

2. EXPERIMENTAL AND CLINICAL DATA THAT FORMED THE BASIS FOR THE AUTOIMMUNE HYPOTHESIS OF ATHEROGENESIS

2.1. Classical Concepts for the Development of Atherosclerosis

In developed countries, cardiovascular diseases are notorious as the main cause of mortality, and atherosclerosis plays an important role in this context. It is, therefore, one of the diseases that has been most extensively studied from various viewpoints in numerous laboratories around the world.

Atherosclerosis is a multi-factorial disease based on the action of various risk factors that become effective on an appropriate genetic background. The disease is characterized by the appearance of mononuclear cells in the vessel wall at certain predelection sites, such as arterial branching points, which are known to be subject to altered haemodynamic stress. With progression of lesions, smooth muscle cells (SMC) from the media immigrate into the intima, where they proliferate and lead to the deposition of extracellular matrix (ECM) proteins, notably collagen fibers. This sequence of events leads to thickening and hardening of arteries (*arteriosclerosis*). *Atherosclerosis* is characterized by the additional formation of foam cells, i.e. macrophages and SMC that have taken up chemically modified, e.g. oxidized low density lipoproteins (oxLDL) via non-saturable scavenger receptors, leading to overloading of these cells with lipids and the eventual deposition of extracellular cholesterol crystals. According to a conventional classical view of atherogenesis, whitish cushion-like lesions, so-called fatty streaks, with a predominance of foam cells constitute the precursors of more severe, rupture-prone, often exulcerated and even calcified lesions, i.e. atherosclerotic plaques.

The classical concepts of atherogenesis do not ascribe a major significance to inflammatory-immunologic processes as possible primary pathogenetic factors. The *"response to injury" hypothesis* [1] originally postulated an alteration of the endothelium (mechanical injury, toxins, free radicals, etc.) as the initiating event leading to endothelial cell (EC) dysfunction, followed by increased permeability, expression of adhesion molecules and release of growth factors and chemotactic factors. As a consequence, platelet aggregation and monocyte adhesion and activation take place, the latter being attracted into the subendothelial space of the intima where they meet with SMC immigrating from the media, followed by foam cell formation, as mentioned above. As the lesion progresses, a fibrotic "cap" and a rupture-prone "shoulder" region characterized by ECM deposition are formed.

Recently, the "response to injury" hypothesis has been complemented and extended by the so called *"athero-ELAM" hypothesis* (endothelial-dependent mechanisms of leukocyte recruitment in atherogenesis) [2, 3]. This hypothesis focuses on endothelial dysfunction as the main initiating factor of atherogenesis. Non-adaptive changes of endothelial structure or function caused by pathophysiological stimuli may lead to localized acute or chronic alterations in the interaction of ECs with cellular and humoral circulating blood components and other layers of the vascular wall. These changes include increased permeability and subsequent oxidative modification of plasma proteins, a hyper-tendency for adhesion of blood leukocytes and a functional imbalance of pro- and antithrombotic factors, growth- and inhibitory factors as well as dilatory and constrictive vasoactive substances.

The *"altered-lipoprotein" hypothesis* [4] postulates an initiating role of chemically-altered lipoproteins, notably oxLDL, that lead to the primary formation of foam cells in the intima. This hypothesis has recently been modified, since it has been shown that only native rather than oxLDL is found in the circulation, and that native LDL transported into the intima through the endothelium is modified (oxidized) and retained there, where it acts as a chemoattractant for monocytes and SMC and is later taken up by these cells, resulting in foam cell formation (*"retention of modified LDL" hypothesis*) [5].

A third classical concept, less significantly supported by experimental and clinical data, is the *"monoclonal SMC proliferation" hypothesis* [6]

which suggests initiation of the disease by clonally-proliferating SMC.

3. PREVIOUS OBSERVATIONS THAT SUPPORT THE ROLE OF INFLAMMATORY-IMMUNE PROCESSES IN THE DEVELOPMENT OF ATHEROSCLEROSIS

An association between inflammatory processes and atherogenesis has been postulated by many authors in the past, but, surprisingly, were ignored for a long time by groups engaged in "classical" atherosclerosis research or considered to be only a secondary phenomenon [7, 8]. Such changes include, the occurrence of granular deposits of immunoglobulins and co-distributed complement components, increased expression of C3b receptors (CR1) and C3b1 receptors (CR3) on macrophages within atherosclerotic lesions, but not in unaltered vessels [9, 10]. However, B cells are only found in very low numbers in various stages of atherosclerotic lesions, and the site of production for these immunoglobulins must, therefore, be sought elsewhere [11]. Various candidates against which these antibodies could be directed have been discussed, among them oxLDL [12] and, as shown by ourselves, certain stress proteins [13].

Other than these humoral immune phenomena, it is now clear that T cells are among the first cells infiltrating the intima of arteries during the earliest stages of atherosclerosis, most probably before monocytes [14]. In an immunohistological study of vascular arterial specimens of young (<35 years) and old (>65 years) patients who died from non-atherosclerosis-associated diseases, we were able to show that the earliest mononuclear cells infiltrating the intima at sites of the development of atherosclerotic lesions were T cells, in contrast to current dogma, rather than macrophages, the latter dominating at later stages together with SMC immigrating from the intima. A majority of these early T cells are CD4+, HLA-DR+ and interleukin-2 receptor+ (IL-2R+), i.e. activated [11, 15]. Other authors were able to show that T cells in late atherosclerotic plaques express the low molecular variant of the "leukocyte common antigen" (CD45RO) and the integrin "very late activation antigen-1" (VLA-1) [16]. Hansson et al. [17, 18], analyzing the rearrangement of T cell receptor (TCR) genes in these latter cells derived from advanced lesions, showed that they represent a polyclonal population rather than displaying restricted T-cell receptor TCR usage. Mosorin et al. [19] have recently confirmed our data in rabbits [20] by showing HSP60 to be the main antigenic candidate against which T cell clones derived from human atherosclerotic plaques are reacting.

Regardless of which antigen these lymphocytes may recognize, it seems unprobable that ECs that aberrantly express major histocompatibility complex (MHC) class II antigens act as primary antigen-presenting cells for T cell sensitization. In our own experiments, we were only able to show MHC class II expression by EC at sites where T cell accumulations, and thus production of gamma-interferon (IFNγ), where present in the intima directly beneath these areas [11]. Therefore, we and others concluded that the expression of MHC class II molecules by EC represents a secondary rather than a primary phenomenon [21, 22]. We originally reasoned that sensitization of T cells takes place at other sites, e.g. the draining lymph nodes. This concept was indirectly supported by the demonstration of increased serum levels of neopterin in patients with atherosclerosis, speaking for a systematic activation of macrophages by IFNγ [23, 24]. However, as will be detailed below, we have recently made the surprising discovery that a whole network of dendritic cells (DC) resembling Langerhans cells in the skin is present in the arterial intima [25], an observation that has completely changed our ideas with respect to the possible *in situ* T cell sensitization in the vascular system.

The large majority of CD3+ in the mononuclear infiltrate in atherosclerotic lesions expresses the TCRα/ß, but an unexpectedly high proportion also expresses the TCRγ/δ [15]. While the latter type of cells only constitutes approximately 1% in peripheral blood, enrichment to 10% and more within early atherosclerotic lesions can be observed. The majority of these latter cells express the TCRγ2 chain, i.e. resembles the TCRγ/δ+ population found in the intestinal mucosa. On the other hand, TCR Vγ9δ2+ cells characteristic of circulating TCRγ/δ+ cells are not proportionally increased in the intima. Furthermore, we were able to demonstrate consider-

able accumulations of T cells at various sites of the *normal* arterial intima of adults and even children and babies, i.e. without concommitant occurrence of atherosclerotic lesions [26]. Together with the above-mentioned accumulations of TCRγ/δ+ cells, notoriously present in the so-called mucosa-associated lymphoid tissue (MALT) compared to other lymphatic organs, e.g. the spleen, this observation prompted us to put forward the hypothesis of the existance of a "vascular-associated lymphoid tissue" (VALT). We hypothesized that the VALT may fulfill a task similar to MALT, i.e. monitoring exogenous or autologous antigenic material that comes into contact with bodily surfaces, in this case that of the vascular system [27]. It later turned out that the observation of an enrichment of TCRγ/δ+ cells may have special importance, since they are known to preferentially react with certain stress proteins (heat shock proteins – HSP) [28], a phenomenon that will be detailed below.

Finally, it was possible to demonstrate on the protein- and mRNA-level that EC as well as leukocytes occurring in atherosclerotic lesions are able to produce a variety of immunological-inflammatory mediators. Among others, these include interleukin-1 (IL-1: EC, SMC, macrophages), tumor necrosis factor α (TNFα: SMC, T cells, macrophages), lymphotoxin (LT: T cells), IL-2 (T cells), IL-6 (EC, SMC, macrophages), IL-8 (EC, macrophages), monocyte-chemotactic peptide-1 (MCP-1: EC, SMC, macrophages) and IFNγ (T cells) [1, 7, 8]. Together, these molecules can modulate the local cellular immune response within emerging atherosclerotic lesions [2]. In addition, growth factors, such as platelet-derived growth factor (PDGF), exert a mitogenic effect on mesenchymal cells and stimulate leukocyte migration. Thus, they play an important role in the maintenance of the immunologic-inflammatory reaction. Normal vascular tissues do not display a high content of PDGF-ß receptors, but in the case of arterial diseases associated with an activation of macrophages and T cells, a considerable expression of such receptors can be observed. The immune reaction within the arterial wall, therefore, seems to entail an increased responsivity to PDGF-ß [1, 2].

Based on these data, we performed a large series of studies in experimental animals and humans that are summarized in the next paragraph, and that finally led to the formulation of our new *"autoimmune" hypothesis* for the development of atherosclerosis.

4. SUMMARY OF THE AUTOIMMUNE HYPOTHESIS OF ATHEROGENESIS

Our initial experiments attempted to identify the antigen(s) that may incite the cellular and/or humoral immune response at the beginning of the development of atherosclerotic lesions. Among the many candidate antigens, microbial constituents, e.g. confronting the immune system in the course of various infections, and oxLDL emerged as the most prominent. Our experimental and clinical data suggest that the earliest stage of atherogenesis consists of an autoimmune reaction against a stress protein, *viz.* HSP60 [13, 29, 30].

HSPs are expressed by prokaryontic and eukaryontic cells constitutively and/or under stress conditions [31, 32]. Physiologically, they fulfill important functions in conjunction with the folding and intracellular transport of proteins. Under stress (e.g. mild heat — hence the name toxins, oxygen radicals, infection, mechanical stress, etc.), some HSPs exert a chaperone function, i.e. associate with other cellular proteins and protect them from denaturation. HSPs are classified into several families according to their molecular mass (Table 1). The main groups are the 100 kD, 70 kD, 60 kD, 40 kD and low molecular weight families. HSPs are phylogenetically highly conserved. Thus, HSP 60 of mycobacteria (mHSP65), *Chlamydiae* (cHSP60), and *E.coli* (GroEL) show over 95% homology on the DNA and protein level. In fact, human HSP60 (hHSP60) and mHSP65 are still approximately 55% homologous [33]. Since HSP60 quantitatively and qualitatively constitute very important antigenic components of bacteria, parasites and even viruses (often contained in the envelope of the latter [34], nearly all humans show humoral and cellular immune reactivity against them. Due to the high degree of sequence homology, this protective immune reactivity (including that derived from vaccinations) may have to be "paid for" by the danger of cross-reactivity with autologous animal or human HSP60 [35].

Our evidence that such an (auto)immune reaction against HSP60 may be instrumental in initiat-

Table 1. The HSP superfamily: Physiological role and possible relation to the immune response of major HSPs

Family	Major members	Important physiological function	Possible role in the immune response
HSP90	HSP90, HSP83	Prevention of steroid receptor binding to DNA; tyrosine kinase phosphorylation	Tumor resistance; autoimmunity
HSP70	HSP70, BiP hsc70, grp78 dnak	Protein folding and unfolding; protein translocation; assembly of multimeric complexes	Immunoglobulin assembly; class II antigen processing; antigen of many pathogens; autoimmunity
HSP60	HSP65, groEL	Protein folding and unfolding; assembly of multimeric complexes	Antigen of many pathogens; autoimmunity
Ubiquitin	Ubiquitin	Protein degradation	Class I – antigen processing; lymphocyte homing; autoimmunity

ing atherosclerosis can be summarized as follows:

- Immunization of normocholesterolemic rabbits with heat-killed mycobacteria or recombinant mHSP led to the emergence of arteriosclerotic lesions, i.e. intimal infiltration by MNC with a preponderance of activated CD4+ T cells, but no foam cells, at sites of the arterial tree known to be predisposed to disease development [36].

- Rats, rabbits and humans express HSP60 in EC at these sites, providing a prerequisite for interaction of specific T cells and antibodies elicited previously by infection or vaccination [15, 19, 26, 37].

- *In vitro*, the same stressors lead to the *simultaneous* expression of HSP60 and adhesion molecules (intercellular adhesion molecule-1 – ICAM-1; endothelial leukocyte adhesion molecule-1 – ELAM-1; vascular cell adhesion molecule-1 – VCAM-1) by EC at the mRNA and protein levels [38]. However, arterial EC seem to be more susceptible than venous EC to the action of various stressors (notably oxLDL), probably based on a lower threshold for the latter due to the prestressing effect of life-long exposure to the higher arterial blood pressure. It is known that venous bypasses of occluded arteries often undergo severe restenosis and "venosclerosis" subsequent to being subjected to the higher arterial blood pressure conditions. We have recently developed a mouse model [39] for carotid bypasses that allows for an indepth study of this issue using appropriate donor (e.g. cytokine or adhesion molecule knock out mice) and recipient combinations, as well as various therapeutic (drugs, antisense oligonucleotide, etc.) interventions [40, 41].
  There is ample evidence that infections may be involved in atherogenesis. Thus, Marek disease virus (MDV), an avian herpes virus leading to neurolymphomatosis in chickens [42], also induces atherosclerosis and CMV [43, 44], herpes virus [45] and *Chlamydia pneumoniae* [46, 47, 48] have been discussed as being associated with the development of human atherosclerotic lesions.
  Our own studies of this problem were based on our long-standing experience with experimentally-induced and spontaneously occurring organ-specific autoimmune diseases in humans and various animal models [49, 50] and our interest in a possible role of an altered lipid metabolism in the decrease of immune reactivity in older age [51].

- The first, presumably autoimmune, step of atherosclerosis that can be induced by three immunizations of rabbits at five week intervals with mHSP65 is still reversible, i.e. subsides after an interval of 32 weeks, while the more severe atherosclerotic lesions induced by feeding a cholesterol-rich diet only or following immunization plus feeding a cholesterol-rich diet do not regress during this period of time [52]. Immunosuppression of rabbits by treatment with an anti-rabbit pan T cell mouse monoclonal antibody in combination with prednisolone (to prevent the

formation of anti-mouse Ig antibodies) inhibits the development of atherosclerosis induced by immunization with mHSP65 [53].

- The mild atherosclerotic aortic lesions that develop in C57BL/6J mice upon feeding a cholesterol-rich diet are aggravated by immunization with mHSP65 [54, 55]. Interestingly, immunization of such mice with chemically-modified LDL prevents the emergence of atherosclerosis. The results of these experiments, which are even more significant when ApoE-/- mice are used [56], proved that (a) immunity to HSP is pathogenic, and (b) an immune reaction to oxLDL may be beneficial, probably by removing oxLDL via immune complex formation.

- As expected, the peripheral blood as well as the arterial lesions induced in rabbits by immunization with mHSP65 are enriched for HSP65-reactive T cells compared to unimmunized controls. It was, however, notable that T cell lines derived from atherosclerotic lesions of unimmunized rabbits fed a cholesterol-rich diet also showed a significantly increased proportion of HSP65 specificity compared to T cell lines derived from the peripheral blood of the same animals [19], pointing to a preferential activation of such cells in the lesions.

- *In vitro* data obtained from examination of arterial and venous cells, including human umbilical vein EC (HUVECs) as well as macrophages of animals and man, showed that HSP60 can be induced in the cytoplasma of these cells by various types of stress factors, e.g. TNFα, H_2O_2, LPS, elevated temperature, or chemical stress [37, 57]. Furthermore, HSP60 is expressed on the surface of stressed cells, thus providing the basis for lysis of these, but not unstressed cells, by human and animal anti-mHSP65 antibodies via complement-mediated cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC) [57, 58]. *In vitro* and *in vivo* experiments in rats involving mechanical stressing of EC have provided evidence for a significant upregulation of HSP60 and adhesion molecule expression under these circumstances [59].
- Studies on the effect of an immunosuppressive treatment performed in *in vitro* experiments showed that treatment with aspirin leads to the induction of HSP60 by ECs, but is also able to suppress the TNFα-induced expression of adhesion molecules (ICAM-1, VCAM-1, ELAM-1), thus potentially inhibiting the interaction of HSP60-specific T cells with their targets [60].

- In a large study of sera from a clinically-healthy group of human volunteers, aged 40–90 years (the so-called "Bruneck Study" [61, 62]), we showed a correlation of anti-mHSP65 antibody titers with the presence of sonographically-demonstrable atherosclerotic lesions in the *A.carotis* [63]. Statistical analyses revealed that these antibodies reflected a risk factor independent of classical risk factors for atherogenesis, except age. Furthermore, it was shown that these antibodies not only react with mHSP65, but show strong cross-reactivity with HSP60 of *Chlamydia pneumoniae* (cHSP60), mHSP65, GroEL and, most importantly, hHSP60 [64]. As mentioned, it was then shown that human affinity chromatography-purified anti-cHSP60 or anti-GroEL antibodies were also able to lyse stressed human EC [58] or macrophages [57] in a complement-dependent fashion or via ADCC. That the mitochondrial protein HSP60 is transported to the cell surface [65] has, in the meantime, been unequivocally corroborated by our collaborators Soltys and Gupta [66], although the possible functional role at that location is still elusive.

- In a recent follow-up study to our work on anti-HSP65/60 antibodies in the sera of the "Bruneck Study", we were able to show that this parameter is very robust, and thus a good indicator of morbidity, but it is also an indicator of mortality, since patients who died during the period from 1990 to 1995 had significantly elevated antibody titers [67].

- Initial attempts to identify mHSP65/HSP60 cross-reactive epitopes recognized by human antibodies using overlapping peptides spanning the whole mHSP65 molecule, revealed three linear epitopes, i.e. at the N-terminus AA 91-105, the C-terminus AA 501-515, and a less well defined AA 171-185 stretch in between [68]. Since antibodies, in con-