Role of the complement system in the pathogenesis of atherosclerotic vascular diseases

PhD Theses

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INTRODUCTION

Cardiovascular diseases, developing from atherosclerosis, are the leading causes of death in Western societies. Atherosclerosis is a progressive disease, which compromises the following separate stages: endothelial dysfunction – fatty streak – intermediate lesion – fibrous plaque – complicated lesion – vulnerable plaque – plaque rupture/erosion. Genetic predisposition and risk factor burden play key role in the development of the process – atherosclerosis is a multifactorial disease. In 1999, Russel Ross was the first, who published that atherosclerosis is an inflammatory disease. The "response to injury" theory on the pathophysiology of atherosclerosis is best acknowledged nowadays. Endothelial dysfunction plays essential role in the initiation of atherosclerosis, while chronic inflammation plays role in the progression of the process. Inflammation is considered as a novel risk factor of atherosclerosis, which is reflected by several inflammatory markers. High sensitivity C-reactive protein (CRP) is the best studied among those, however the role of certain complement proteins (C3, C4) were also investigated.

There are some special cases of vascular lumen stenosis, with different pathomechanism than those described in primary atherosclerosis. The progression might be accelerated, with different macroscopic and microscopic findings. The balance of lipoprotein mediated and immunologic mechanisms differ from primary atherosclerosis. Restenosis following vascular surgery is considered as one of those special cases. Restenotic lesions are diffuse, concentric and do not contain a lipid core. During eversion carotid endarterectomy, the atherosclerotic plaque and the intimal layer are removed, exposing a new surface on the vessel wall and during cross clamping of the carotid artery ischemia/reperfusion (I/R) occurs. This triggers an intense inflammatory response and thrombosis, which leads to increased production of inflammatory cytokines and growth factors. This leads to accumulation of vascular smooth muscle cells and monocytes in the lesion. Thrombocytes adhere and become activated on the injured surface, thereby enhancing the inflammatory response. This finally leads to neointimal hyperplasia and thickening, vascular remodelling and finally to restenosis.

The complement system is a part of the innate humoral immune system. It has three physiologic activities: defending against microbial infection, bridging innate and
adaptive immunity, and disposing of immune complexes and the products of inflammatory injury. Complement is able to recognize potentially harmful foreign and altered-self structures, thus plays an essential role in the maintenance of immunohomeostasis. Components of complement are present in the circulation in high amounts, as inactive proteins. Once initiated, the complement system responds in cascade-like activation against the activator agent. Activation and response is not antigen-specific and occurs in the absence of T-cells or antibodies. Lysis of target cells, opsonisation and enhancement of the inflammatory reaction are the main consequences of activation.

The complement system plays an important role in the pathogenesis of atherosclerosis. No signs of complement activation can be detected in intact arteries, while there is an intense activation within atherosclerotic lesions. Complement activation is more intense in vulnerable or ruptured plaques. Anaphylatoxins, opsonins and the membrane attack complex (MAC) of complement mediate numerous cell-specific effects to the smooth muscle cells, monocytes and T-cells in the atheroma. Moreover, they enhance surface thrombogenity, the attraction of leukocytes and apoptosis within the lesion.

I/R injury is characterized by the presence of activated polymorphonuclear leukocytes, production of free-oxygen radicals, release of cytokines and eicosanoids. I/R injury promotes attraction, activation, adhesion and migration of leukocytes and production of inflammatory mediators, which cause organ injury. Complement activation supports the inflammatory response in I/R injury by production of anaphylatoxins (C3a, C5a) and by MAC mediated cell lysis.
AIMS

1. To study the association between the progression of severe coronary artery disease (CAD) and serum C3 levels

C3 might be a novel risk factor for atherosclerosis. The serum concentration of C3 correlates with several risk factors; such as age, body mass index (BMI), blood pressure, cholesterol and triglyceride levels, and history of smoking or hypertension. Recently we also found significant positive correlation between C3 levels, BMI and serum lipid concentrations in healthy subjects. According to the studies of Muscari et al. high C3 levels are able to predict acute myocardial infarction (AMI) in men. Our purpose was to examine the possible role of C3 in assessing risks for the severe complications of atherosclerosis; focusing also on women, who are often left out of studies on cardiovascular diseases. The study was performed in patients with pre-existing severe coronary artery disease, who underwent coronary artery bypass surgery.

2. To study the association between early restenosis following carotid endarterectomy, serum C3 levels and the genotype of the mannose-binding lectin gene (MBL2)

Early restenosis complicates carotid endarterectomy (CEA) in 13% of cases in the first two years following surgery. In contrast to primary atherosclerosis, this process is characterized by neointimal hyperplasia, due to cytokine and growth factor overproduction. Recently in a prospective study, we demonstrated that homozygous carriers of the wild type (A) alleles of the mannose-binding lectin (MBL2) gene have significantly higher risk to develop early restenosis after eversion CEA compared to carriers of the defect (B, C, or D) alleles of MBL2. We hypothesized that postoperative changes of C3, a complement APR – in contrast to other non-complement APRs – may specifically reflect the involvement of complement in this inflammatory process. Our objective was to investigate the associations of C3 with the development of an early restenosis following CEA, considering MBL2 genotypes. In addition, we investigated the associations of other known non-complement acute phase reactants (CRP, haptoglobin, and α2-HS Glycoprotein [α2HSGP]) with early restenosis.
3. To study the association between early restenosis following carotid endarterectomy, serum C1-inhibitor (C1-INH), MBL-associated serine protease-2 (MASP-2) levels, and the genotype of MBL2

Homozygotes for the normal allele of MBL2 gene have higher risks to develop an early restenosis after eversion CEA. These patients have significantly higher serum MBL concentrations, compared with the carriers of the variant alleles of MBL2. This leads to the assumption, that an intact lectin pathway might be required for restenosis. During the activation of the lectin pathway, MBL-MASP-2 complexes are able to cleave C4 and activate the cascade. C1-INH – a serine protease inhibitor – is the main inhibitor of lectin pathway activation. According to the literature, patients with unstable angina pectoris tended to have higher C1-INH levels compared with patients with stable angina pectoris. In contrast, a different study showed no difference in C1-INH levels in patients with prior AMI compared with healthy controls. The association of MASP-2 levels with the presence and progression of atherosclerosis has not been studied yet.

Our objective was to investigate the associations of C1-INH and MASP-2 levels with the development of an early restenosis following CEA. In addition, we investigated how MBL2 genotypes affect these associations.

4. To study the presence and mechanism of complement activation following CEA and its relation to the time of I/R injury

I/R injury is characterized by an intense inflammatory response to the temporary ischemia and return of the blood supply of the tissues. Increasing evidence shows, that complement has an essential role in I/R injury and targeting complement might be effective in the reduction of tissue damages caused by I/R injury. However, most of these data comes from animal experimental models and only scarce data is available on in vivo complement activation due to I/R injury in humans. During CEA, cross clamping of the carotid artery is performed, which might trigger I/R injury and possibly complement activation.

Our primary aim was to detect the presence and to investigate the mechanism of early complement activation in patients who underwent CEA. We also investigated the relation of complement activation to the time of the cross clamping of the carotid artery. In addition, we investigated complement activation in patients with stenting of the carotid artery – where no cross clamping occurs.
METHODS

Prospective study of patients with severe CAD
266 (67 women and 199 men, aged 41 to 85 years) Hungarian patients with severe CAD were included in the study. The diagnosis was based on clinical signs of stable or unstable angina pectoris, typical ECG changes and signs of severe stenosis confirmed by coronary angiography. Between 1995 and 1996, all patients received an aorto-coronary bypass graft (ACBG) by an open-heart surgery in the National Institute of Cardiology, Budapest. The serum and DNA samples were collected 6 months after the coronary bypass operation, when none of the patients was showing clinical signs of acute inflammation. In December 2000, we sent a questionnaire to the patients or contacted their GPs to gather data on the occurrence of new events after the operation (death of the patient, new AMI, ACBG, PTCA, stroke, carotid surgery and peripheral arterial disease). One-hundred and eighty-two healthy controls (100 women and 82 men, Hungarian blood donors or volunteers, aged 20 to 79 years) took part in this study. The study was approved by the local ethical committee, and all subjects gave informed consent.

Prospective study of patients who underwent carotid endarterectomy
In this study, we included 64 patients (21 female and 43 male, aged between 43 and 79 years) with severe carotid atherosclerosis who underwent eversion type CEA and were followed-up at the Department of Cardiovascular Surgery, Semmelweis University, Budapest between October 2000 and March 2003. Clinical history and physical examination were taken in all patients focusing with high priority on cardiovascular risk factors (CAD, stroke, transient ischaemic attack (TIA), diabetes mellitus, peripheral artery disease, hypertension, hyperlipidaemia, smoking status). Patients with malignancy and vasculitis were excluded from the study. The indication for CEA was in accordance with the current American Heart Association guidelines. Eversion CEA was performed by six different surgeons, the postoperative outcome did not differ among them. All patients were followed-up for at least 1 year (median 13.8, interquartile range 12.3-19.0 months), while they had medical check-up with carotid duplex scan (CDS).
examinations at 6 weeks (median 5.7, interquartile range: 4.6-8.0 weeks), 7 months (median 6.8, interquartile range 6.2-7.9 months) and 14 months (median 13.8, interquartile range: 12.3-19.0 months). Restenosis at 14 months was considered significant, when restenosis of the operated internal carotid artery exceeded 50%. In 10/64 (15.63%) patients, restenosis developed during the follow-up period, which is comparable to the literature. Blood samples from antecubital veins were drawn at four different time intervals; preoperatively and 4 days, 6 weeks and 14 months post-surgery. Fifty-nine healthy controls (27 women and 32 men, Hungarian blood donors or volunteers, aged 32 to 61 years) took part in this study. The study was approved by the Ethical Committee of Semmelweis University; all patients gave informed consent.

**Prospective, observational study of patients who underwent carotid endarterectomy**

We included 16 patients (9 male and 7 female, aged 41-84 years), who underwent eversion type CEA at the Department of Cardiovascular Surgery, Semmelweis University, Budapest between January and May 2007 in the study. Only patients without clinical signs or medical documentation of acute or severe chronic infection, autoimmune disease, sepsis or malignancy were included. Clinical history and physical examination were taken in all patients focusing with high priority on cardiovascular risk factors (CAD, stroke, TIA, diabetes mellitus, peripheral artery disease, hypertension, hyperlipidaemia, smoking status). Indication of CEA was in accordance with the American Heart Association guidelines. The time of clamping of the carotid artery was recorded during surgery. Ten patients (7 males and 3 females, aged 50-75 years) who underwent percutaneous transluminal carotid artery balloon angioplasty and stenting (CAS) at the same department between March and May 2007 were studied as patient controls. Indication for CAS was in accordance with the American Heart Association/American Stroke Association guidelines. Blood samples were serially drawn from antecubital veins prior CEA/CAS and immediately after, 1, 4, 8 and 24 hours following surgery/intervention. All patients gave informed consent and the study was approved by the Ethical Committee of the Semmelweis University, Budapest.
Laboratory methods
Serum and EDTA-anticoagulated plasma specimens were collected in the studies. Serum samples were allowed to clot for 2 hours prior separation and were deep frozen at -20°C. EDTA-anticoagulated plasma samples were immediately (within 1 hour) separated and were deep frozen at -80°C. Total genomic DNA was extracted from white blood cells using a simple salting out method from EDTA-anticoagulated samples stored at -20°C. Determination of complement- and acute-phase proteins (C3, C1-INH, MASP-2 CRP, haptoglobin, α2HSGP), serum lipids (total cholesterol, triglycerides), complement activation products (C1rsC1INH, C4d, C3a, SC5b-9), MBL2 genotypes and allotypes of the C3 gene were done after appropriate melting of the deep frozen samples.

Clinical studies
CDS examinations (ATL Ultramark 9 HDI system) were performed by an experienced radiologist preoperatively and at 14 months post-surgery. The common, internal and external carotid arteries on both sides were examined in the standard fashion. The diagnostic criteria for internal carotid artery stenosis and restenosis were based on peak systolic velocities and end diastolic velocities as well as internal carotid artery/common carotid artery ratios. The degree of stenosis was defined between 0% and 100% - where 0% indicated an intact vessel, while 100% indicated a total occlusion.

Statistical analysis
Statistical analysis was performed with Prism for Windows 3.0 and 4.02 (GraphPad Software, San Diego, CA) and SPSS for Windows 10.0, 11.5 and 13.0.1 (SPSS Inc., Chicago, IL) statistical software products. All statistical analyses were performed two-tailed and p<0.05 was considered as significant.
RESULTS

High C3 levels are associated with the progression of CAD
Serum C3 levels were higher in women with severe CAD compared with healthy controls, adjusted for age, BMI, systolic and diastolic blood pressure, total cholesterol, triglyceride and CRP concentrations (p=0.021). There was no association between C3 levels and C3*F allotypes. We found no significant differences in C3 levels according to the smoking habits in the patients. In women with severe CAD, concentrations of C3 did not correlate with age, BMI, total cholesterol and triglycerides. High C3 levels (C3 ≥ 1.8 g/L) predict new cardiovascular events following ACBG surgery, the odds ratio (OR) was 4.1 (95% confidence interval (CI): 1.23-13.61, p=0.0249). The value of OR adjusted for age, BMI and smoking was 4.8 (95% CI: 1.137-20.070, p=0.033).

High C3 levels are associated with early restenosis following carotid endarterectomy; partially dependent of MBL2 genotypes
C3 levels at 4 days following surgery did not increase compared with the preoperative values – probably due to the consequence of complement consumption. Later, an increasing tendency was observed during the follow-up period and C3 reached significantly (p<0.001) higher levels at 14 months post-surgery compared to the baseline values, but only in patients with MBL A/A genotypes (p<0.001). We found that patients with early restenosis had remarkably higher C3 levels in blood samples taken at 14 months, compared to the patients who did not develop significant restenosis (p=0.0175). A strong significant positive correlation was found between C3 concentrations and the degree of restenosis (r=0.3311, p=0.0075), which was only significant in homozygous carriers of the wild-type alleles of the MBL2 gene (r=0.4904, p=0.0021). We found that high C3 levels were associated with the development of restenosis even after adjusting for MBL2 genotype, age and gender (OR: 4.8924, 95% CI: 1.146-21.4739, p=0.0354). We found no significant associations between the non-complement acute-phase proteins (CRP, haptoglobin, α2HSGP) and early restenosis following CEA.
Low C1-INH levels predict early restenosis following CEA in homozygous carriers of the wild-type alleles of MBL2

C1-INH levels significantly increased at 4 days post-surgery compared with the preoperative values (p<0.01), while it decreased to baseline at 6 weeks post-surgery level (p<0.01), independent of MBL2 genotypes. We found that patients with significant restenosis had lower C1-INH levels in blood samples taken at 6 weeks (p=0.0052) and at 4 days (p=0.0277), compared with the patients who did not develop significant restenosis. The degree of restenosis based on CDS results at 14 months correlated with serum C1-INH levels at 6 weeks (r= -0.3415, p=0.0058), which was only significant in patients with MBL A/A genotypes (r= -0.5044, p=0.0015). Patients with low C1-INH levels had significantly higher CDS values at 7 months (p<0.01) and low C1-INH levels are associated with high CDS values during the follow-up period at high statistical significance (p<0.0001). Patients with low C1-INH levels (C1-INH<115%) at 6 weeks post-surgery have 6.93 (95% CI: 1.55-31.02, p=0.0113) times higher risk to develop an early restenosis in 14 months, compared with the other patients independent of age and gender. The predictive value of low C1-INH levels was significant and much more pronounced in patients with the MBL2 A/A genotype, the OR for the development of an early restenosis was 13.97 (95% CI: 1.95-100.21, p=0.0087). We found no significant association between MASP-2 levels and early restenosis following CEA, levels of MASP-2 showed an increasing tendency at 4 days and decreased significantly at 6 weeks post-surgery (p<0.01).

Early complement activation follows CEA, which is associated with the time of I/R injury

C3a increased sharply immediately after surgery (p<0.001) and remained higher compared with the baseline values and patients with stenting at 1 and 4 hours following CEA (p<0.05). We observed a modest increase in SC5b-9 levels immediately after surgery compared to the baseline in patients with CEA (p<0.05). Levels of C1rsC1INH and C4d did not change significantly during the study period in either patient group. The absence of C3a elevation did not depend on the MBL2 genotypes. We found a strong positive correlation between peak C3a levels and the time of clamping in patients who underwent CEA (r=0.5921, p=0.0200). In both patient groups CRP levels
significantly increased at 24 hours post-surgery/intervention compared with the baseline values (p<0.01 for both). We did not find significant changes in C1rsC1INH, C4d, C3a or SC5b-9 levels in the first 24 hours following carotid artery stenting.
CONCLUSIONS

The major findings of the study were:

1) High C3 levels predict the progression of severe coronary artery disease in women, who underwent coronary-artery bypass graft surgery, independently of other risk factors of atherosclerosis. C3 might be a novel specific chronic inflammatory marker of the progression of atherosclerosis.

2) High C3 levels are associated with the presence of an early restenosis following eversion carotid endarterectomy, partially depending on MBL2 genotype. The complement system and C3 might be directly involved in the pathogenesis of restenosis. Monitoring of C3 levels might be useful in the follow-up of patients with carotid endarterectomy.

3) Low C1-inhibitor levels predict the development of an early restenosis following eversion carotid endarterectomy. The predictive value is only significant in homozygous carriers of the wild-type alleles of the MBL2 gene. Serum MASP-2 levels are not associated with early restenosis following carotid endarterectomy. Determining C1-inhibitor concentrations and MBL2 genotypes might be useful to assess prognosis following carotid endarterectomy.

4) Early complement activation follows eversion carotid endarterectomy. The role of the alternative pathway is suggested in the initiation. The degree of complement activation is associated with the time of ischaemia/reperfusion injury. Further studies are required to understand the role of early complement activation in the disease process.
SCIENTIFIC PUBLICATIONS

Publications summarized in the dissertation:

   **IF: 3,796 (2004)**

   **IF: 2,803 (2006)**

   **IF: 6,883 (2006)**

   **IF: 4,768 (2006)**

Other publications:

   **IF: 7,667 (2005)**

   **IF: 2,355 (2006)**

   **IF: 2,803 (2006)**

   **IF: 4,768 (2006)**

13

IF: 4,768 (2006)


IF: 2,328 (2006)


IF: 5,012 (2006)


IF: 8,829 (2006)


IF: 6,555 (2006)


IF: 2,524 (2006)


IF: 3,811 (2006)


IF: 2,747 (2006)