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Review

Homocysteine, heat shock proteins, genistein and vitamins in ischemic stroke — pathogenic and therapeutic implications

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Stroke is one of the most devastating neurological conditions, with an approximate worldwide mortality of 5.5 million annually and loss of 44 million disability-adjusted life-years. The etiology of stroke is often unknown; it has been estimated that the etiology and pathophysiology remains unexplained in more than 40% of stroke cases. The conventional stroke risk factors, including hypertension, diabetes mellitus, smoking, and cardiac diseases, do not fully account for the risk of stroke, and stroke victims, especially young subjects, often do not have any of these factors. It is very likely that inflammation, specific genetic predispositions and traditional risk factors interact with each other and may together increase the risk of stroke. Inflammatory and immune responses play important roles in the course of ischemic stroke. Hyperhomocysteinemia (hcy) is considered a modifiable risk factor for stroke, possibly through an atherogenic and prothrombotic mechanism. Both genetic and environmental factors (e.g., dietary intake of folic acid and B vitamins) affect homocysteine level. Identification of the role of hcy as a modifiable risk factor for stroke and of HSPs as regulators of the immune response may lead to more effective prevention and treatment of stroke through dietary and pharmacological intervention. Dietary modification may also include supplementation with novel preventive compounds, such as the antioxidative isoflavones — genistein or daidzein.

Key words: ischemic stroke, homocysteine, heat shock proteins, genistein

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INTRODUCTION

Cerebral ischemia causes different physiological, biochemical and morphological changes in the brain. Growing evidence suggests that inflammation plays an important role in the development of cardiovascular and cerebrovascular diseases (Saikku *et al*., 1988; Wick *et al*., 2001). Hyperhomocysteinemia contributes to the pathogenesis of ischemic stroke by causing atherogenesis *via* endothelial damage and coagulation abnormalities (Lentz, 1997; Coppola 2000). One of the important mechanisms involved in the development of vascular lesions leading to ischemic stroke could be an immune response to heat shock proteins (HSPs) (Mandal, 2004).

The better understanding of the role of hyperhomocysteinemia and HSPs in atherosclerosis should lead to new approaches for the prevention and treatment for all forms of vascular diseases. In this review, we discuss the role of homocysteine and heat shock proteins in the pathogenesis of ischemic stroke and the potential role of genistein and vitamins in stroke prevention.

STROKE PATHOGENESIS — HOMOCYSTEINE IN ISCHEMIC BRAIN STROKE

Homocysteine is an amino acid normally absent in diet, but its metabolism and level may depend on dietary factors, such as a lack of folates (Selhub *et al*., 1999). Homocysteine is involved in the transfer and metabolic cycle of the methyl group. The only source of homocysteine in human is demethylation of methionine (Finkelstein, 1990), while excess of homocysteine is eliminated through remethylation to methionine and transsulfurylation pathway. The physiological range of total plasma homocysteine concentration is approx. 515 μM and it increases with age (Ueland *et al*., 1993). Hyperhomocysteinemia can be classified in three classes, depending on severity: 16–30 μM is mild, 31–100 μM is intermediate, and over 100 μM is severe (Welch *et al*., 1998; Kaul *et al*., 2006). Genetic research has demonstrated that one of the main causes of increased homocysteine concentration are mutations of genes coding for enzymes of the metabolic pathway of the methyl group. The main enzymes involved in homocysteine metabolism are cystathionine β-synthase (CBS), methionine synthase (MS), and methylenetetrahydrofolate reductase (MTHFR). CBS catalyzes the conversion of homocysteine to cystathionine, MTHFR generates 5-methyltetrahydrofolate, the methyl donor used for homocysteine remethylation by MS. Two know polymorphisms of the *MTHFR* gene are associated with diminished activity of the enzyme (Weisberg *et al*., 1998; Jakóbkiewicz-Banecka *et al*., 2005). Vitamins of the B group are another diet-dependent factor influencing homocysteine metabolism, since cobalamin is a cofactor of MS and pyridoxine of CBS.

While the first suggestions concerning homocysteine atherogenicity were published in the 1960s and were based on observations of vascular lesions in children with inborn errors of homocysteine metabolism (McKully, 1969), epidemiological studies have confirmed the significance of hyperhomocysteinemia as one of the most important risk factors for a number of cardiovascular

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Abbreviations: CBS, cystathionine β-synthase; HSP, heat shock proteins hcy, hyperhomocysteinemia, MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase

diseases, including atherosclerosis of coronary, cerebral and peripheral blood vessels (Welch *et al.*,1998; Kaul *et al.*, 2006), as well as ischemic brain stroke (Sawuła *et al.*, 2009). Even though hyperhomocysteinemia alone would not be as important as conventional risk factors (e.g., smoking or hypertension), a synergistic effect between homocysteine concentration and classic risk factors has been suggested (Terwecoren *et al.*, 2009). It has been estimated that the increase in homocysteine concentration of 5 μM increases the risk of atherosclerosis by 60–80%. Since even a mild increase in homocysteine concentration might have significant clinical implications, it is of uttermost importance to select a suitable diagnostic technique (Sawuła *et al.*, 2008).

Several hypotheses have been put forward concerning homocysteine pathogenicity, which was first attributed mainly to homocysteine thiolactone, T-Hcy (Jakubowski, 2001). T-Hcy is a reactive form of homocysteine and it may bind to proteins, decreasing their activity (Jakubowski, 2007). Concentration of T-Hcy depends on total homocysteine concentration and it has been demonstrated that disruptions of one of the key stages of homocysteine metabolism results in an increase in T-Hcy concentration (Chwatko *et al.*, 2007). T-Hcy may exhibit its pathogenicity through processes such as disruption of fibrinogen structure leading to abnormal fibrinolysis, inhibition of lysine oxidase leading to disruption of collagen synthesis, or binding to albumin leading to decrease of albumin transport capabilities in the serum. The proposed biological mechanisms of homocysteine pathogenicity include impaired endothelial function (Lentz, 1997), disruption of cholesterol and triglyceride biosynthesis (Werstuck *et al.*, 2001), activation of monocytes (Zeng *et al.*, 2003), and promotion of vascular smooth muscle cell (VSMC) proliferation (Tsai *et al.*, 1994). The proposed biochemical mechanisms include production of reactive oxygen species leading to auto-oxidation (Welch *et al.*, 1997), hypomethylation (Lee *et al.*, 1999), protein homocysteinylation (Jakubowski 2001), and binding to nitric oxide leading to nitrosylation (Perna *et al.*, 2003).

Auto-oxidation has been deemed especially plausible, since homocysteine contains a free sulfhydryl group of strong redox properties. Auto-oxidation of homocysteine leads to generation of a disulfide and reactive oxygen species, which may also be created as a result of formation of a mixed disulfide of homocysteine and other free sulfhydryl amino acids (Hayden, 2003). Reactive oxygen species may initiate lipid peroxidation, thus starting a process leading to disruption of endothelial function.

Homocysteine may lead to hypomethylation, because it can combine with adenosine to form *S*-adenosylhomocysteine (SAH), which is a very strong inhibitor of cellular methylation able to significantly affect DNA synthesis. This mechanism has been demonstrated to play a major role in endothelial cells, while homocysteine does not appear to inhibit DNA synthesis in aortic smooth muscle cells or fibroblasts (Jiang *et al.*, 2005). This mechanism is independent of the free sulfhydryl group and the inhibitory effect on endothelial cells is exhibited at clinically relevant concentrations, potentially making it a major factor in homocysteine-induced atherosclerosis.

Homocysteine may directly affect nitric oxide synthesis by inhibition of enzymes in this metabolic pathway, which may account for another mechanism of suppression of endothelial cell proliferation or viability. Homocysteine may directly interact with nitric oxide synthase and indirectly lead to accumulation of asymmetric dimethylarginine (ADMA). Nitric oxide is synthesised in the endothelial lining of blood vessels by nitric oxide synthase (Becker *et al.*, 2005) and it is crucial for maintaining proper vascular function. It modulates blood flow by relaxation of smooth muscles in vessels, exhibits anti-proliferation and anti-coagulation action, as well as counteracts LDL oxidation. Nitric oxide is essential for proper functioning of blood vessels and plays a significant role in counteracting atherosclerosis. It may be a major factor in regulation of oxidative processes, which seem to be of significance in the development of a number of vascular diseases (Ignarro *et al.*, 1989). A decrease in nitric oxide concentration may have a dramatic impact on vascular function (Papapetropoulos *et al.*, 1997). Because of this, inhibitors of nitric oxide synthesis may be implicated in the development of atherosclerosis and brain stroke. One of such inhibitors is ADMA (Blackwell, 2010). ADMA exhibits strong affinity to all forms of nitric oxide synthases and it leads to dislodgement of L-arginine, which is a substrate in NO synthesis, from the enzymatic active site, thus leading to a total inhibition of the synthesis of nitric oxide and inducing synthesis of reactive forms of oxygen (Kocaman, 2009). An additional mechanism of ADMA action may be induced by overproduction of LOX1 receptor, which is the main receptor for oxidated LDL in vascular wall cells. Nitric oxide deficiency is a risk factor in atherosclerosis and it could be a marker of the progressive development of atherosclerotic plaque. It has been demonstrated that chronic homocysteine administration decreases nitric oxide levels, thus inducing oxidative stress, but this effect may be counteracted by concurrent administration of folic acid, another dietary factor influencing enzymes involved in homocysteine metabolism (Kolling *et al.*, 2011).

STROKE PATHOGENESIS — HEAT SHOCK PROTEINS AS REGULATORS OF THE IMMUNE RESPONSE

Heat shock proteins (HSP) play an important role in protecting cells against damage due to various stress factors. In addition to their protective function HSP are also associated with the pathogenesis of atherosclerosis. Numerous research groups have provided evidence that HSP may be a potential risk factor in the development of atherosclerosis and related cardio and cerebrovascular diseases, such as stroke (Pockley, 2003; Benarroch, 2011; Chen *et al.*, 1997). Early after cerebral ischemia, protein synthesis in the brain is generally suppressed, but specific genes are expressed and their corresponding proteins may be synthesized; they include heat shock proteins and amyloid precursor protein (Kogure *et al.*, 1993). Cerebral ischemia produces many endogenous ligands and cytokines that cause an immune response. Leukocytes, including neutrophils and monocytes, are activated and recruited to initiate processes of containment, removal and repair. It is believed that both inflammation and immunological mechanisms play an important role in the pathogenesis of atherosclerosis, including autoimmune response against heat shock proteins (Lindsberg *et al.*, 2003). Most conventional risk factors for atherosclerosis, such as high blood pressure and metabolic disorders, increase heat shock protein expression in endothelial cells, smooth muscle cells and macrophages, the main cellular components of atherosclerotic plaques. Another risk factor involved in the development of atherosclerosis are chronic infections with pathogens, causing endothelial damage due to cross-reactivity with autologous proteins (Elkind, 2010; Lindsberg *et al.*, 2003; Benarroch, 2011).

HSP comprise proteins belonging to several families, depending on their molecular weight (Mehta, 2005).

Their expression can be induced or assisted by heat treatment, exposure to oxygen radicals or cytokines, ischemia, surgical stress, bacterial or viral infection. It has been shown that increased levels of anti-HSP60, anti-HSP65 and anti-HSP70 antibodies in the plasma can be correlated with the development and severity of atherosclerosis (Xu *et al*., 1993; Xiao *et al*., 2005).

It has been shown that the levels of IgG and IgM anti-HSP 65 and anti-HSP 70 antibodies are significantly increased in the sera of stroke patients just after stroke onset (Gromadzka *et al.* 2001). An increased level of anti-HSP IgG was identified as an independent risk factor for stroke. This suggests a possible causal relationship between chronic humoral immunity to HSP and development of vascular lesions leading to ischemic stroke.

In order to evaluate the role of HSP70.1 protein in ischemia, the mitochondrial apoptotic pathway was analyzed using *HSP70.1* knockout (KO) and wild-type (WT) mice (Lee *et al*., 2004). It has been demonstrated that a markedly reduced expression of HSP70 induced a larger infarction volume after focal ischemia and more abundant apoptotic cell death after transient focal ischemia. Moreover, the increased apoptosis in the KO mice was associated with increased release of cytochrome *c* into the cytoplasm and subsequent activation of caspase-3. Those increased apoptotic features were more prominent in the cortex, where the HSP70 protein expression was obviously upregulated (Lee *et al*., 2004).

The 70-kDa heat-shock protein (HSP70) family is a group of chaperones assisting in folding, transporting, and assembling of proteins in the cell (Georgepulous *et al*., 1993). HSP70 induction protects cells from lethal insults other than heat shock (Kabakov *et al*., 1995). In vivo protection by HSP70 has been reported in models of myocardial (Marber *et al*., 1995) or cerebral ischemia (Rajdev *et al*., 2000). However, the biochemical cascades underlying this protective role of HSP70 are still uncertain, and individual functions of *HSP70.1* and *HSP70.3* genes are not understood (Lee *et al*., 2004).

Brain ischemia alone induces HSPs. Immunocytochemically, these HSPs are seen primarily in neurons but also to some extent in glia and endothelial cells after experimental ischemia (Chen, 1997). Although a neuroprotective role for these proteins in brain ischemia remains speculative, the temporal profile of the expression of HSP follows the pattern of selective vulnerability in ischemic brain, being seen first in the cortex and thalamus, and later in the resistant region of the dentate granule cells after graded global ischemia. Furthermore, HSPs, mainly the 70-kDa form, is consistently upregulated in tolerant brains, an expression pattern compatible with the "dose response" and "time window" characteristics of ischemic tolerance (Lindsberg, 2003; Mandal *et al*., 2004).

One of the important areas of current research is the role of HSPs in the association between infection and atherosclerosis. Based on their observations, an autoimmune hypothesis of atherogenesis has been proposed (Wick *et al*., 2001). HSP60 is expressed by endothelial cells of stressed arteries and since microbial and human HSP60 are characterized by a high degree of sequence homology, the very mechanisms of immunity against microorganisms may be responsible for endothelial cell damage and early atherosclerosis.

As pointed out by Lindsberg *et al*., infectious agents, mainly viruses, have been implicated in atherogenesis for several decades (Lindsberg *et al*., 2003). Anti-HSP antibodies may be produced in response to infection and this possibly provides a link between atherosclerosis and chronic infection. Several studies aimed at finding a link between atherosclerosis and HSPs pointed at the significance of *Chlamydia pneumoniae*, a Gram-negative intracellular bacterium that is distributed worldwide. The serological evidence of *C. pneumoniae* infection has been associated with myocardial infarction (Saikku *et al*., 1988). In 40 studies *C. pneumoniae* bacteria were detected in coronary and carotid plaques but only rarely or not at all in normal vessel walls using such diverse techniques as polymerase chain reaction, immunohistochemistry and electron microscopy (Grayston, 2000).

C. pneumoniae can induce proatherogenic and prothrombotic changes involving activation of the transcription factor NF-κB (Godzik *et al*., 1995). Aspirin inhibited *C. pneumoniae*-induced NF-κB activation and chlamydia growth. *C. pneumoniae*-reactive T lymphocytes were detected in carotid plaques, and cross-reactivity between human and chlamydial HSP60 may also play a role in the atherosclerotic process (Mosorin, 2000). Chlamydial HSP60 induced the production of TNF-α and matrix-degrading metalloproteinases by plaque macrophages, mechanisms that may contribute to plaque rupture and thrombosis. Clinical data support the claim that the presence of *C. pneumoniae* in carotid stenoses increases local thrombogenicity and the risk of infarction, but not all results confirm this hypothesis (Gibbs *et al.* 2000).

Long-standing risk factors and chronic infectious diseases, possibly in conjunction with genetic predispositions, may lead to gradual activation of circulating mononuclear cells and subsequent entry of these cells to subendothelial/perivascular region, which in turn may aggravate the proinflammatory and procoagulant action of endothelial cells effects (Lindsberg *et al*., 2003). Infections with microbes such as *C. pneumonia* may actively prime this process in larger arteries and contribute to maturation of atherosclerotic plaques (Mehta *et al*., 2005) Inflammatory cells are always present in these plaques and respond to further systemic stimuli by releasing proteases and procoagulant factors, which can trigger plaque rupture and thromboembolism (Perttu *et al*., 2003).

However, the role of chronic infection and inflammation in stroke pathogenesis is still incompletely defined. Future studies need to address whether inhibition of inflammation can reduce the risk of ischemic stroke to offer new approaches for treatment. Humoral immunity to HSPs is common in stroke patients and elevated levels of anti-HSP antibodies could be triggering factors for stroke. There is growing evidence that atherosclerosis may be an inflammatory and possibly an immune disorder (Mehta *et al*., 2005). Due to the high degree of sequence homology between human and microbial HSPs, anti-microbial HSP antibodies produced in response to infection cross-react with HSPs on endothelial cells stressed by classical risk factors for atherogenesis and stroke (Mandal *et al*., 2004). Prospective studies consistently show an increasing risk of stroke along with increasing levels of systemic inflammatory parameters at baseline, although the overall strength of the association is mostly moderate (Jickling *et al*., 2012).

Although HSPs help individual cells to survive, as they perform important functions, it is not clear if for the organism as a whole the misguided mechanisms of HSP could also contribute to the pathogenesis of fatal diseases. HSPs appear to be risk factors that may act in cooperation with conventional risk factors and genetic predispositions and are neither necessary nor sufficient for disease development. However, it is not yet established whether lowering of inflammatory indexes lowers stroke risk, and any causal role of these parameters in

stroke pathophysiology is unproven. More comprehensive understanding of the role of HSPs in atherosclerosis and stroke is likely to lead to new approaches of the prevention and treatment for all forms of cardiovascular disease (Pockley, 2003; Benarroch, 2011; Gromadzka *et al*., 2001).

STROKE THERAPY — GENISTEIN AND VITAMINS

Atherosclerotic plaque formation is associated with the presence of immune cells (Hansson *et al*., 2006). Cells at the site of the development of atherosclerotic plaque are subjected to stress conditions and chaperone proteins are over-expressed locally. One of the types of antibodies found in the atherosclerotic plaque are antibodies directed against these proteins. As a result, the chaperone proteins exhibit an immunogenic effect in the atherosclerotic plaque, which leads to a faster inflammatory process and acceleration of atherosclerotic plaque accumulation. On the other hand, HSPs play a protective role towards a variety of enzymes, thus potentially reducing homocysteine level.

The main enzymes of homocysteine metabolism comprise vitamin cofactors, mainly of the B group (Finkelstein, 1998). It has been demonstrated that partial loss of activity of these enzymes can be efficiently compensated by increased concentration of cofactors and it has been suggested that supplementation with B vitamins could lower homocysteine concentration in individuals with certain genetic polymorphisms (Hadithi *et al*., 2009). It is worth pointing out that for several years B vitamins have been added to cereal in certain European countries and in the United States, since during processing these products are depleted of important biologically active components, including vitamins. However, the lowering of the homocysteine level in high risk groups achieved through vitamin B supplementation seems insufficient. Thus gaining further insight into the mechanism of pathogenesis of ischemic brain stroke and searching for other preventive factors gains significance. Based on an animal model of singlet oxygen-induced cerebral stroke it has been suggested that one of such factors could be genistein — a compound present in soy and other isoflavone-rich legumes (Trieu 1999). Isoflavones regulate lipoprotein metabolism, increase HDL and reduce LDL and VLDLD level, lower platelet activity, and improve vascular reactivity. Isoflavones (such as genistein or daidzein) are characterized by two benzene rings connected by a three-carbon chain. This class is sometimes denoted as C6-C3-C6. It has been suggested that isoflavones are responsible for the anti-atherosclerotic benefits of soy (Wang, 1994). Due to their chemical properties, isoflavones are potent antioxidants — they are capable of neutralization of free radicals and reduction of platelet aggregation through inhibition of lipid peroxidation or stimulation of formation of nitric oxide in vascular endothelium. The presence of oxidized LDL and the oxidative stress resulting from immunological reactions lead to reduction of the amount of NO produced by endothelium (Davignon *et al*., 2004). As a result, the immune system is stimulated and immunological reaction appears in the atherosclerotic plaque. Genistein could be used to prevent this mechanism of ischemic stroke, especially considering the fact that isoflavones exhibit only weak estrogenic activity and have minimal side effects. Research in hypertensive rats has demonstrated that isoflavones, including genistein, inhibit growth and DNA synthesis of aortic smooth muscle cells in these stroke-prone animals, thus proving the potential of genistein in prevention of the basic mechanism of vascular change underlying development of atherosclerosis (Pan, 2001). Further research in hypertensive rats has confirmed the significance of the preventive value of genistein in stroke through attenuation of NADPH oxidase expression and activity (Xu, 2004). Potential use of genistein has also been studied in a murine model of metabolic syndrome — genistein improves insulin sensitivity and restores renal function in fructose-fed rats (Palanisamy, 2008) and counteracts the negative effects of fructose overfeeding in respect to blood pressure and cholesterol level (Palanisamy, 2012).

CONCLUDING REMARKS

Due to the high incidence and mortality, stroke is one of the most intensively studied diseases. Recent studies provide new information on stroke pathogenesis, including the significance and mechanism of action of homocysteine and ADMA, but numerous questions remain unanswered, such as the role of heat shock proteins in stroke patients. Novel diagnostic and preventive measures are developed, such as genistein supplementation, which seems to offer several benefits in prevention of vascular diseases. All these tools and data are required to efficiently combat stroke and other diseases of cardiovascular origin.

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