Dysregulated RANK Ligand/RANK Axis in Hyperhomocysteinemic Subjects
Effect of Treatment With B-Vitamins

Marit S. Nenseter, PhD; Thor Ueland, PhD; Kjetil Retterstøl, MD, PhD; Ellen Strøm, MS; Lars Mørkrid, MD, PhD; Sverre Laadaas, MD, PhD; Leiv Ose, MD, PhD; Pål Aukrust, MD, PhD; Kirsten B. Holven, PhD

Background and Purpose—Homocysteine has been linked to increased risk of ischemic stroke and other cardiovascular events. Matrix degradation and inflammation play an important role in these disorders, and we have demonstrated increased levels of matrix-degrading enzymes and inflammatory cytokines in hyperhomocysteinemic individuals. Recent studies suggest that RANK ligand (RANKL) through interaction with its receptor RANK can modulate matrix degradation and inflammation. The present study aimed to examine the role of the RANKL/RANK axis in hyperhomocystinemia.

Methods—RANKL/RANK was measured on protein or mRNA level before and after B-vitamin supplementation in hyperhomocysteinemic individuals. We also examined the in vitro effects of soluble RANKL in peripheral blood mononuclear cells from hyperhomocysteinemic individuals.

Results—Our main findings were: (1) compared to peripheral blood mononuclear cells from controls, cells from hyperhomocysteinemic individuals had significantly higher gene expression of RANKL and RANK; (2) folic acid treatment for 6 weeks in an open, uncontrolled study significantly reduced gene expression of RANKL/RANK in peripheral blood mononuclear cells from these individuals; (3) compared to placebo, treatment with folic acid, vitamin B12, and vitamin B6 for 3 months in a randomized, double-blind trial significantly lowered serum levels of soluble RANKL in hyperhomocysteinemic individuals; and (4) in vitro, soluble RANKL markedly increased the release of matrix metalloproteinase-9 and inflammatory cytokines from peripheral blood mononuclear cells in hyperhomocysteinemic subjects.

Conclusions—Our findings suggest a dysregulated RANKL/RANK axis in hyperhomocysteinemic subjects. Based on their role in atherogenesis, this enhanced expression of RANKL and RANK could contribute to the increased risk of cardiovascular disease in hyperhomocystinemia. Moreover, treatment with B-vitamins may have beneficial implications for plaque stability in these individuals. (Stroke. 2009;40:241-247.)

Key Words: B-vitamins ■ homocysteine ■ inflammation ■ RANK ■ RANKL

Data from epidemiological studies suggest that raised homocysteine levels are associated with an increased risk of ischemic stroke, myocardial infarction, and other vascular disorders. However, several large clinically controlled trials have now shown that lowering homocysteine with vitamin-B therapy does not reduce risk of cardiovascular disease (CVD) or mortality among subjects with known CVD. Regarding stroke, however, a recent meta-analysis showed that folic acid supplementation can effectively reduce the risk of stroke in primary prevention, with a particularly marked effect in those who achieved a decrease in the homocysteine concentration of ≥20%. Thus, the role of homocysteine in CVD is still debated, and the precise mechanism by which hyperhomocystinemia is related to atherogenesis remains unclear.

Increasing evidence supports the involvement of inflammation and matrix degradation in the pathogenesis of atherosclerosis and plaque destabilization. We have previously shown that hyperhomocysteinemic subjects are characterized by raised serum levels of markers of inflammation and matrix degradation like IL-6, epithelial cell-derived neurophil-activating peptide (ENA)-78, and matrix metalloproteinase...
(MMP)-9, potentially promoting atherogenesis and plaque instability.12–14 The receptor activator of nuclear factor-kappa B (RANK) and osteoprotegerin are members of the tumor necrosis factor receptor superfamily.15–16 Osteoprotegerin is a critical modulator of RANK ligand (RANKL) activity through its action as a decoy receptor for RANKL, thereby blocking interaction of RANKL with its receptor RANK.15–16 The RANKL/RANK axis has been shown to have pleiotropic effects on bone metabolism, endothocrine function, and the immune system, and recently this axis has also been implicated in the development of atherosclerotic disorders.16–18 Thus, RANKL has been detected in atherosclerotic lesions, potentially promoting inflammation, endothelial cell activation, and matrix degradation.19 In addition, a novel role of RANKL as a vascular permeability factor was recently reported.20 Furthermore, raised serum levels of soluble RANKL (sRANKL) have been reported in patients with heart failure,19,21,22 and a recent large-scale epidemiological study supports a role for RANKL in CVD by the ability of sRANKL to predict vascular risk in apparently healthy individuals.23 In this latter study, the authors also found a strong association between serum levels of sRANKL and forthcoming stroke and transient ischemic attack. Moreover, symptomatic carotid plaques have been shown to express high levels of osteoprotegerin, further supporting the involvement of osteoprotegerin/RANKL/RANK axis in cerebrovascular diseases.

The aim of the present study was to examine the RANKL and RANK in hyperhomocysteinemia. Several approaches were used. First, we compared gene expression of RANKL and RANK in peripheral blood mononuclear cells (PBMC) from hyperhomocysteinemic subjects and matched healthy normohomocysteineemic volunteers. Second, we studied the effect of folic acid therapy on these parameters in an open, uncontrolled study. Third, the effect of folic acid, vitamin B₁₂, and vitamin B₆ (TrioBe) on sRANKL levels was examined in a randomized placebo-controlled, double-blind trial. Finally, the in vitro effect of sRANKL on PBMC from hyperhomocysteinemic individuals was investigated.

Methods

Subjects and Study Design

Hyperhomocysteinemic Subjects and Healthy Control Subjects

Fourteen adults aged younger than 70 years with hyperhomocysteinemia (fasting plasma total homocysteine concentration >15 μmol/L at screening) were consecutively recruited at the Lipid Clinic and the Department of Medical Biochemistry, Rikshospitalet University Hospital, and at Department of Clinical Chemistry, Ullevål University Hospital, Oslo, Norway (Table 2). The patients were randomized in a double-blind fashion to receive therapy with TrioBe (Recip AB) or placebo. Inclusion criteria were women and men aged 18 to 70 years of age, plasma homocysteine levels ≥15 μmol/L, and written consent. Exclusion criteria were the presence of cancer or other coexisting serious disease, reduced renal function, treatment with B-vitamins or untreated vitamin B₁₂ deficiency, treatment with phenobarbital, phenytoin, methotrexate, or trimethoprim, pregnant or breast-feeding women, or inability to follow the protocol, as judged by the investigator. The study was designed to examine inflammatory parameters in hyperhomocysteinemia. The allocation of patients to treatment groups was performed by randomization using the method of randomly permuted blocks. The randomization list and sealed envelopes were prepared by specialized staff at Recip AB, who was not involved in the study. All study investigators, personnel, and participants were unaware of the treatment assignments. The participants were randomly allocated to receiving either TrioBe (folic acid, 0.8 mg; cyanocobalamin, 0.5 mg; and pyridoxine hydrochloride, 3.0 mg; 1 tablet per day; Recip AB) or an identical-appearing placebo tablet (1 tablet per day; Recip AB) for 3 months. Compliance as judged by pill count of returned unused pills was 90%, 86% to 95%, and 88%, 84% to 91% (P = 0.11) in the TrioBe and placebo groups, respectively. Assessment of dietary intake was performed by self-administered food questionnaire (SmartDiet; Lipid Clinic)24 at baseline and after 3 months of treatment. Thirty-eight of the 42 participants completed the study (20 in the TrioBe and 18 in the placebo group). The reasons for not completing the study were: a new cardiovascular event (ie, stroke; n = 1); side effects (n = 1); start of new medication during the study period (n = 1); and stopping to take pills (n = 1).

Ethics

All parts of the study were approved by the Regional Committee of Medical Ethics. The intervention studies were approved by The

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<th>Table 1. Characteristics of Participants</th>
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<td>P-homocysteine, μmol/L</td>
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Data are given as median (25–75 percentiles). Number of individuals indicated by n. P indicates plasma, S, serum.

Vitamin B₁₂, B₆, and Folic Acid (TrioBe) Therapy

Forty-two adults 18 to 70 years of age with hyperhomocysteinemia (fasting plasma total homocysteine concentration >15 μmol/L at screening) were recruited at the Lipid Clinic and the Department of Medical Biochemistry, Rikshospitalet University Hospital, and at Department of Clinical Chemistry, Ullevål University Hospital, Oslo, Norway (Table 2). The patients were randomized in a double-blind fashion to receive therapy with TrioBe (Recip AB) or placebo. Inclusion criteria were women and men aged 18 to 70 years of age, plasma homocysteine levels ≥15 μmol/L, and written consent. Exclusion criteria were the presence of cancer or other coexisting serious disease, reduced renal function, treatment with B-vitamins or untreated vitamin B₁₂ deficiency, treatment with phenobarbital, phenytoin, methotrexate, or trimethoprim, pregnant or breast-feeding women, or inability to follow the protocol, as judged by the investigator. The study was designed to examine inflammatory parameters in hyperhomocysteinemia. The allocation of patients to treatment groups was performed by randomization using the method of randomly permuted blocks. The randomization list and sealed envelopes were prepared by specialized staff at Recip AB, who was not involved in the study. All study investigators, personnel, and participants were unaware of the treatment assignments. The participants were randomly allocated to receiving either TrioBe (folic acid, 0.8 mg; cyanocobalamin, 0.5 mg; and pyridoxine hydrochloride, 3.0 mg; 1 tablet per day; Recip AB) or an identical-appearing placebo tablet (1 tablet per day; Recip AB) for 3 months. Compliance as judged by pill count of returned unused pills was 90%, 86% to 95%, and 88%, 84% to 91% (P = 0.11) in the TrioBe and placebo groups, respectively. Assessment of dietary intake was performed by self-administered food questionnaire (SmartDiet; Lipid Clinic)24 at baseline and after 3 months of treatment. Thirty-eight of the 42 participants completed the study (20 in the TrioBe and 18 in the placebo group). The reasons for not completing the study were: a new cardiovascular event (ie, stroke; n = 1); side effects (n = 1); start of new medication during the study period (n = 1); and stopping to take pills (n = 1).
RNA from each sample was reverse transcribed by TaqMan high-capacity reverse transcription reagent kit (Applied Biosystems). For quantitative real-time reverse-transcription polymerase chain reaction amplification, sequence-specific polymerase chain reaction primers were designed using the Primer Express software version 1.5 (Applied Biosystems) for RANK (forward primer [FP]: 5’-CCCCGTGGCAGCTCAACAAG-3’; reverse primer [RP]: 5’-GCATTTGTCCGTGAGGAAA-3’) and RANKL (FP: 5’-GTGCAAAAGGAAATTACAACATATCGT-3’; RP: 5’-AACACATGAGCCATCCACAT-3’). The ß-actin was used as a housekeeping gene for normalization (Applied Biosystems).

**Ellyume Immunoassays**
Concentrations of sRANKL were quantified by Enzyme Immunoassays from Biomedica GmbH (Vienna, Austria). Concentrations of MMP-9, ENA-78, and IL-6 were measured by Enzyme Immunoassays (R&D Systems).

**Routine Laboratory Assays**
Concentrations of P-homocysteine were measured on the Abbott IMx analyzer, S-folate and S-vitamin B12 were measured on the Wallac AutoDELfia analyzer, and P-total cholesterol, P-LDL-cholesterol, P-HDL-cholesterol, P-triglycerides, and P-creatinine were measured using the Modular-P platform (Roche).

**Statistical Analysis**
Data are given as median (25–75 percentiles) if not otherwise stated. Data from patients and controls and differences in changes between groups were compared by the Mann-Whitney U test. For categorical data, Pearson χ² test was used. Changes in variables within groups were analyzed by the Wilcoxon signed-rank test. Multiple linear regression analysis was used to test dependent and independent variables. Probability values (2-sided) were considered significant at values of P<0.05.

**Results**
**PBMC Expression of RANKL and RANK in Hyperhomocysteinemic Subjects and Healthy Control Subjects**

Table 1 shows characteristics of the control and patient populations. No significant differences were observed for age, total cholesterol, or creatinine, whereas low-density lipoprotein cholesterol was significantly higher in hyperhomocysteinemic patients compared to controls (Table 1). The expected differences were observed with regard to homocysteine, folate, and vitamin B12 (Table 1).

When comparing PBMCs from the hyperhomocysteinemic individuals and healthy controls, we found markedly enhanced gene expression of RANKL and its corresponding receptor, RANK, in cells from those with hyperhomocysteinemia (Figure 1). Moreover, although the groups were imbalanced with regard to smoking and statin use, with higher proportions of these characteristics in those with hyperhomocysteinemia (Table 1), there were no significant differences in gene expression of RANK and RANKL between smokers (n=9) and nonsmokers (n=5; P=0.74 and P=0.84, respectively). However, statin users showed higher gene expression of RANKL and RANK (n=5) compared to statin nonusers (n=5; P=0.072 and P=0.014, respectively). Eight of the hyperhomocysteinemic subjects were included in an open, uncontrolled folic acid study, and folic acid supplementation for 6 weeks resulted in a significant decrease in plasma levels of homocysteine (from 27, 22–40 μmol/L to 10, 9–11 μmol/L, n=8; P<0.02) and a significant increase in...
Effect of TrioBe Therapy on Soluble RANKL in Hyperhomocystinemia

RANKL also exists in circulation as a biological active molecule. Therefore, we next measured serum levels of sRANKL in subjects with hyperhomocystinemia and explored the effect of folic acid, vitamin B12, and vitamin B6 (TrioBe) supplementation on this mediator in a randomized, double-blind, placebo-controlled trial. Characteristics of the participants are given in Table 2, showing no significant differences between the treatment groups. Whereas 3 months of TrioBe treatment increased serum levels of folate from 7.6, 4.3–7.7 nmol/L to 35, 25–49 nmol/L, n = 8; P < 0.02). Notably, these changes during folic acid supplementation were accompanied by a decrease in mRNA levels in PBMCs for both RANKL and RANK (Figure 1 A, B).

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Effect of RANKL In Vitro on PBMCs From Hyperhomocystinemia

RANKL has been shown to induce MMP activation and inflammation, and we have previously found that hyperhomocysteinemic subjects have increased plasma levels of MMP-9 as well as several inflammatory mediators such as IL-6 and ENA-78. To explore any possible pathogenic role of sRANKL in hyperhomocystinemia, we next examined the in vitro effect of sRANKL on the release of MMP-9, IL-6, and ENA-78 in PBMCs after culturing for 24 hours. The cells were isolated from 10 hyperhomocysteinemic subjects (plasma levels of homocysteine 18, 17–22 μmol/L; serum levels of folate 8.5, 7.0–11.0 nmol/L). As shown in Figure 3, recombinant sRANKL significantly enhanced the release of MMP-9, ENA-78, and IL-6, with particularly marked effects on the 2 latter mediators.
Discussion

A role for the RANKL/RANK axis in atherogenesis and plaque destabilization has recently been reported by several groups, potentially also involving promotion of stroke and transient ischemic attack.16–23 In the present study we demonstrate that hyperhomocysteinemic individuals are characterized by enhanced gene expression of RANKL and its receptor RANK in PBMCs as well as increased serum levels of the soluble form of RANKL. We also show the ability of folic acid and B-vitamins to downregulate these mediators in hyperhomocysteinemic individuals. RANKL has been found to exert inflammatory effects in various cell types, and in the present study we show that sRANKL potently induced the release MMP-9 as well as the inflammatory cytokines ENA-78 and IL-6 in PBMCs from hyperhomocysteinemic subjects. Taken together, these findings may suggest that a dysregulated RANKL/RANK axis could be involved in the pathogenesis of hyperhomocystinemia-related CVD, and that B-vitamins may have potential implications for plaque stabilization in these individuals.

Previously, enhanced RANKL and RANK levels have been reported in patients with chronic heart failure, and several studies have demonstrated increased expression of these tumor necrosis factor-related mediators in atherosclerotic disorders.19–23 In the present study we demonstrate also that hyperhomocysteinemic individuals are characterized by increased expression of these mediators with enhanced expression of both the ligand (RANKL) and its corresponding receptor (RANK) in PBMCs as a major finding. Previously, RANKL has been detected within atherosclerotic plaques from abdominal aortas and carotid arteries in humans. We have recently shown enhanced expression of RANKL and RANK in both clinical and experimental atherosclerosis with particularly high levels in those with unstable disease, potentially contributing to the transition from a stable to an unstable plaque phenotype.19 Our findings in the present study suggest that RANKL and RANK should be added to the list of inflammatory mediators with relation to atherogenesis that are increased during hyperhomocysteinemia. Moreover, several epidemiological observations have linked hyperhomocysteinemia to increased risk for stroke and, interestingly, high RANKL levels have been shown to predict forthcoming cerebrovascular events in healthy subjects.23 It is therefore tempting to hypothesize that the association between hyperhomocysteinemia and enhanced levels of RANK/RANKL also could be of importance for the hyperhomocysteinemia-related increase in cerebrovascular disorders.

Remodeling of advanced atherosclerotic lesions and changes in plaque morphology are multifactorial processes, in which MMPs are suggested to play important roles.26–28 Previously, we have showed an altered MMP-9/tissue inhibitors of matrix metallo-proteinases (TIMP-1) balance in hyperhomocysteinemic individuals, which may contribute to the increased risk of ischemic stroke and other cardiovascular diseases in these subjects.14,25 We have also reported increased levels of the CXC chemokine ENA-78 and IL-6 in hyperhomocysteinemic subjects, potentially contributing to systemic and vascular inflammation in these individuals.12,13 RANKL has been shown to induce matrix degrading and inflammatory effects in various cells, and in the present study we show that sRANKL is a potent inducer of MMP-9, ENA-78, and IL-6 in PBMCs from hyperhomocysteinemic individuals. If this RANKL-mediated interaction also is operating in vivo in hyperhomocysteinemic individuals, then it could contribute to the matrix degrading and inflammatory and proatherogenic phenotype in these individuals, and in those with established atherosclerosis, these inflammatory interactions could potential promote plaque destabilization with subsequent development of transient ischemic attack, stroke, and myocardial infarction. It could be argued that a high concentration of RANKL was used in the in vitro experiment as compared with serum levels of this ligand. However, although we have no data, it is not inconceivable
that such concentrations could exist within an inflammatory microenvironment such as within an atherosclerotic lesion consisting of several RANKL-expressing cells.

An important finding in the present study was that supplementation with folic acid and B-vitamins reduced gene expression of RANKL and its receptor RANK in PBMCs, as well as lowered serum levels of sRANKL. The recently reported improvement in stroke mortality observed after folic acid fortification helps to reduce deaths from stroke.29 These findings are consistent with the hypothesis that folic acid fortification contributes to the increased risk of CVD in these individuals, such as smoking and statin use, and the role of RANKL/RANK in hyperhomocysteinemia-related CVD should be further investigated in larger study populations.

In conclusion, although there were some limitations, the present study suggests that hyperhomocysteinemic subjects are characterized by a dysregulation in the RANKL/RANK axis that at least partly may be restored by B-vitamin supplementation. Based on their role in atherogenesis, matrix degradation, and inflammation, this enhanced expression of RANKL and RANK in hyperhomocysteinemic subjects could contribute to the increased risk of CVD in these individuals, underlining the importance of restoration by B-vitamins.

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Disclosures
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References


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