Ph.D. Thesis

Lipid and homocystein metabolism and oxidative stress in chronic renal failure patients

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1. Introduction and aims

In end stage renal failure (ESRD) atherosclerosis is accelerated. Till now this complication plays leading risk factor in morbidity and mortality of those patients independently of development of dialysis techniques.

There are many well known risk factors of atherosclerosis and arteriosclerosis. In this work we focused on follows:

1.1. Specific and scavenger LDL receptors.

The specific LDL receptors which bound native LDL are expressed on the surface of various cell types, such as fibroblasts, hepatocytes, lymphocytes, monocytes etc., while the scavenger LDL receptors which bound modified LDL are expressed mainly on macrophages.

Binding of native LDL to its receptor initiates cellular events: 1. Inhibition of de novo synthesis of LDL receptor. 2. Stimulation of acethyl-cholesterol-acyl-transferase enzyme activity, and 3. Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase activity which is responsible for endogen cholesterol synthesis, leading to a decrease in cholesterol content of cells.

Binding of modified LDL to its receptor release of apoE will occur. ApoE induces cholesterol transport from cells. Any disturbances in native or scavenger LDL receptor expressions or functions will lead to increased formation of foam cells.

Very few information considering expression and functions of both native and scavenger LDL receptors are available in ESRD patients, in spite of the high risk of these patients for atherosclerosis.

1.2. Fatty acid composition of serum lipids and lipoproteins

Hyperlipoproteinaemia is one of the main risk factor of atherosclerosis. In ESRD patients, lipid abnormalities such as enhanced triglyceride and LDL, and decreased HDL concentrations are well demonstrated. It is also well known that hyperlipidaemia alters fatty acid distribution in various lipids. In those cases a decrease in polyunsaturated fatty acid (PFA) concentrations, and an increase in monounsaturated fatty acid (MUFA) concentrations, among them palmitoleic acid are common changes.

However, there is no data considering the fatty acid profile in ESRD patients, though many data showed the advantageous effects of n-3 PUFA (e.g. fish oil) supplementation on progression of renal diseases.

1.3. Oxidative stress, lipids and neutrophils

Reactive oxygen radicals induced reactions such as lipidperoxidation plays an important role in the pathomechanism of atherogenesis. These free radicals are chemically very reactive and their counteraction with lipids and lipoproteins resulted in injury of arterial wall.

In hemodialysis (HD) patients enhanced lipidperoxidation is due to many factors such as biocompatibility of dialysis membrane, activated neutrophils, and these dialysis-induced activation of neutrophils produce high amounts of reactive oxygen species (superoxide anion, O_2^-, hyrdoxyl etc.).

Recently, it was demonstrated that increased TG and Chol levels in circulation resulted in enhenced O_2^- production by neutrophils in hyperlipoproteinaemic patients. Furthermore, free fatty acids are able to modify O_2^- production by neutrophils in vitro, and in vivo, and their effects depends on the chain length and degree of unsaturation. For example, arachidonic acid enhances, while EPA, DHA attenuate O_2^- production by neutrophils.

In ESRD patients PUFA concentration in free fatty acid fraction of plasma is low, TG and LDL-Chol levels are elevated and oxidative processes in neutrophils are enhanced, however the relationships between these parameters are unkown.

1.4 Oxidative stress and antioxidants

Role of Vitamin E (RRR-α-tocopherol): Oxidative stress is prevented by several antioxidants such as antioxidant enzymes (SOD, catalase, glutathion peroxidase) and non-enzymatic antioxidants such as Vitamin E, free thiols etc. In HD patients decreased erythrocyte antioxidant enzyme activities were demonstrated. A loss in the amounts of the water soluble antioxidants (Vitamin C, free thiol-containing compounds) is due to hemodialysis treatment, while the restriction in diet might contribute to decreased concentrations of lipid-soluble antioxidants (e.g. Vitamin E). Therefore, in HD patients enhanced oxidative stress is concomitant with decreased antioxidant capacity.

Recently, it was demonstrated that antioxidant activity of HDL is due to an enzyme named to paraoxonase (PON-1). PON-1 is a 43 kD glucoprotein binding to N-terminal end of apoA1 in HDL. PON-1 has three fenotypes (due to glutamine-arginine exchange) with different activity (AA, AB, BB). Antioxidant property of PON-1 is due to its ability to hydrolyze lipidperoxides in LDL particle. Distribution of PON-1 phenotypes might be influenced by ESRD, however no date concerning this is known.

1.5. Intracellular free calcium ($[Ca^{2+}]_i$ *) concentrations in neutrophils*

In calcium-dependent signal transduction mechanisms calcium is an important second messenger. These type of signal transduction to work properly, the intracellular free calcium levels must be kept low (50-100 nM) in resting cells. When $[Ca^{2+}]_i$ in resting state is elevated alterations in cell functions were observed (e.g. hyperlipidaemia, atherosclerosis, and ageing). In most calcium-dependent signaling sources of calcium liberation are intracellular pools and extracellular space as well. This latter requires normal calcium-phosphate homeostasis. In CRF patients disturbed calcium-phosphate metabolism, and hyperparathyroidism are well known alterations.

Previously, in was demonstrated that rHuEpo treatment normalized the originally elevated $[Ca^{2+}]_i$ in platelets of HD patients. However, $Ca^{2+}]_i$ in neutrophils and parameters which might alter it in HD patients has not been studied, though alterations in neutrophils functions are well demonstrated.

1.6. Hyperhomocisteinaemia in ESRD patients

It is very frequent, that in many patients suffering from coronary artery diseases, and after myocardium infarct had no classical risk factors of atherosclerosis. During the last decades, epidemiological studies demonstrated that hyperhomocysteinaemia (HHC) is a new and independent risk factor of this disease.

It is known that parallel to decrease in renal functions homocystein (Hcy) concentration increased progressively. Factors leading to high frequency of HHC in ESRD patients are not well known. Since in normal renal function only 1 % of total Hcy can be found in urine.

In the treatment of HHC folic acid and Vitamin B12 which are cofactors in folate-cycle are used, however, contradictory results are available concerning the optimal dose of folate, mainly in ESRD patients.

Hyperhomocysteinaemia and trace elements (cobalt and nickel) in ESRD patients.

Trace element concentrations are disturbed in ESRD patients. These disturbances might influence processes in which trace elements are involved. It was demonstrated that cobalt and nickel play role in methionin-homocystein metabolism. In animals cobalt and nickel supplementation ameliorated homocystein status. However, human results are not known.

3. Aims

1. To study the specific and scavenger LDL receptor expressions and functions in monocytes of HD patients.

2. Determination of fatty acid composition in free fatty acids and total lipid extracts in plasma of HD patients, and study relationships between fatty acids, oxidative burst in resting neutrophils, and serum lipid parameters.

3. Determinations of antioxidant Vitamin E concentrations, PON-1 activity and phenotype distributions in HD patients.

4. Determination of intracellular free calcium levels in neutrophils, and parameters which might influence it (such as rHuEpo therapy, anaemia, time in dialysis treatment, calcium-phosphate metabolism, biocompatibility of dialysis membrane).

5. Determination of homocystein concentrations, polymorfism of methylene-tetrahydrofolate reductase, serum cobalt, nickel, Vitamin B_{12} and folic acid concentrations, and relationships between measured parameters.

3. Methods

Cell separations: neutrophils were separated from heparinized blood of patients by density gradient centrifugation by method of Boyum. Monocytes were separated by method of Kumagai.

LDL isolation: LDL was separated from fasting blood of healthy volunteers (20-30ys) by KBr density ultracentrifugation. Protein content of samples was determined by method of Lowry.

[^{125}J]LDL preparation: was performed by method of Sheperd using ^{125}J isotop. Free ^{125}J was removed by gel filtration from LDL.

Biding and intracellular degradation of [^{125}J]LDL: measurements were performed using native [^{125}J]LDL and [^{125}J]- acLDL. Binding of LDL was determined at 4 °C, while intracellular degradation at 37 °C. Specific binding of labeled LDL was expressed as ng/10^6 cells.

Endogen cholesterol synthesis: was performed by method of McNamara. Monocytes were incubated with native LDL in the presence of 2-[^{14}C]Na acetate (Izinta, Budapest). Lipids were extracted and saponified. Incorpotated radioactivity of samples was determined. Cholesterol production was expressed as pmol[^{14}C]Na acetate/hour/10^6 monocytes.

Secretion of Apo E: Monocytes were incubated with 50 ug/ml acLDL. Supernatant was collected and concentrated. ApoE was measured by nephelometry.

Formation of foam cells: Monocytes were treated with Oil Red O (BDH Chemicals) lipid-dye. Cholesterol deposits were counted by microscopy.

Extraction of lipids and determination of fatty acids: Lipids were extracted from fasting plasma by method of Blight. Free fatty acids were separated by thin layer chromatography. Fatty acids were methylated and determined using gas-chromatography coupled to mass selective detector (GC-MSD - Hewlett Packard, 5970).

Paraoxonase activity: was determined by using paraoxon (O,O-dietil-O-p-nitrofenilfoszfát; Sigma Chemical Co.) substrate by spectrophotometry (412 nm). Enzyme activity was calculated by the use of molar extinction coefficient. One unit enzyme will liberate 1 nmol 4-nitrofenol/ min from substrate.

Distribution of PON-1 phenotypes: was determined by double substrate method. Phenotypes were determined from measured paraoxonase, arylesterase, and salt stimulated enzyme activities.

Determination of lipidhydroperoxids: was performed from the extracted lipids by using potassium iodide and spectrophotometry.

Determination of conjugated dien formation: Extracted lipids were redissolved in cyclohexane (Merck). Optical density of samples was measured at 234 nm.

Determination of lipidperoxidation: Thiobarbituric acid reactive substances (TBARS) were measured by spectrofotometric method.

Superoxide anion production: was determined by SOD sensitive reduction of Cytochrome C using method of Babior.

Determination of intracellular free calcium: was performed by method of Tsien using Quin 2/AM fluorescent dye.

Determination of Vitamin E: was performed by HPLC using γ-tokoferol as internal standard and fluorescent detection.

Determination of homocystein: was performed by commercial kit of Bio-Rad.

Determinations of Vitamin B_{12} and folic acid: were performed by commercial kits of Abbott using Axsym equipment.

Determination of MTHFR C677T polimorfism: was performed using PCR.

Determination of cobalt and nickel: was performed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry).

Satistical methods:

SAS Windows 6.11 program was used for statistical analysis (mean, standard deviation). Statistical significance was determined by paired or unpaired T test. Correlation coefficients were calculated by correlation and regression analysis. Significance was $p<0,05$.

4. Results and discussion

4.1 Specific and scavenger LDL receptor functions in monocytes of HD patients:

It was found that specific LDL receptor expression is lower while scavenger LDL receptor expression is higher in monocytes of HD patients than in controls. Binding rate (V_{max}: 107,8±13,6 vs. 209,7±53,5 nd/106 cells) and degradation of native LDL decreased, however its binding constant (K_d) was unchanged. At the same time binding rate (V_{max}: 1312±598 vs. 42,5±5,5 ng/106 cells), binding constant and degradation rate of modified LDL (acLDL) increased. There was no difference in cholesterol synthesis rate by resting monocytes of HD patients, but apoE secretion also decreased (2,8±0,4 vs. 9,4±1,2 ug/12h/10^6 cells, $p<0,001$). It was concomitant with increased number of cholesterol deposits (11,2±2,3 vs. 2,8±0,3 deposit/cells, $p<0,001$).

In conclusion, an increase in scavenger LDL receptor expression, a decrease in apoE secretion and increased foam cell formation were observed in monocytes of HD patients. Decreased apoE secretion shows that scavenger LDL receptor function is altered, which resulted in enhanced foam cell formation. All of these changes together with lipid abnormalities might play role in atherogenesis of dialysis patients.

4.2. Fatty acid composition of serum lipids and oxidative stress in HD patients.

It was found that progression of chronic renal failure affects fatty acid composition of plasma lipids. In predialysis patients no significant changes were demonstrated. In HD-treated patients amounts of polyunsaturated fatty acids decreased (in free fatty acids: 9.1 vs. 15.8 %; in total lipids: 26.6 vs.35.6%), while ratio of saturated to monounsaturated fatty acids was influenced by severity of hypelipoproteinaemia and cardiovascular complications. However, among polyunsaturated fatty acids the relative deficiency in the amount of essential linoleic fatty acid was common alteration (HD-nor: 17.1±2.8; HD-CAD:20.1±2.8; Control: 26.5±0.9 %, $p<0.01$).

Significant relationship between superoxide anion production of neutrophils and serum lipid parameters (O_2^-/Chol: $r=0.5$, $p<0.025$; O_2^-/TG: $r=0.5$, $p<0.02$), and polyunsaturated fatty acids (O_2^-/PUFA: $r=-0.66$, $p<0.001$) were observed. These relationships suggest that deficiency in

PUFA might be due partially to oxidative degradation. Another hand, relationships between O_2^- production by neutrophils and lipid parameters, demonstrated here for HD patients, might suggest a common relationship which exsist not only in hyperlipidaemia but in other oxidative stress-related states.

Another change in fatty acid composition of plasma lipids with increasing serum lipid levels is an increase in concentration of monounsaturated fatty acid (MUFA) of palmitoleic acid (4.38±2.5 vs. 1.77±0.7%, $p<0.01$). Similar increase in concentration of palmitoleic acid was observed in HD patients as well (4.33±2.5%). However in HD patients concentration of palmitoleic acid did not correlate with serum Chol ($r=0.37$ vs. $r=0.88$), TG ($r=0.01$ vs. $r=0.51$) concentrations, and O_2^- production of neutrophils ($r=0.063$ vs. $r=0.68$) then that were demonstrated in hyperlipidaemic patients. On the basis of these results it might be supposed that the mechanism leading to increase in palmitoleic acid concentration in HD patients differ from that of hyperlipidaemic patients.

Another important differences among HD patients and hyperlipidaemic patients were the more higher O_2^- production by neutrophils and the more higher TBARS levels in various lipids, suggesting that the enhanced oxidative stress in HD patients might be due to other factors (such as uremic toxins), too.

4.3. Vitamin E concentrations and PON-1 activity in HD patients

Alterations in both antioxidants were demonstrated in HD patients. Vitamin E normalized to either serum TG or Chol levels were significantly lower than in controls (Vit E/TG: 4.74±0.86 vs. 5.72±0.94, $p<0.001$; Vit E/Chol: 14.0±5.98 vs. 35.3±8.0, $p<0.0001$) suggesting a deficiency in Vitamin E.

HDL-associated PON-1 activity was also depressed in HD patients (101.36±30.2 vs. 188.05±58.96 U/L, $p<0.001$), and it was not due to decreased HDL and/or apoA1 concentrations, and fenotype distributions of PON-1 which did not differ from controls (AA: 66.67 vs. 66.68%; AB:31.62 vs. 26.67%; BB: 1.71 vs. 6.67%). This decreased PON-1 activity might be due inhibition effects of uremic toxins or altered distribution of HDL subspecies.

In conclusion, decreased PUFA concentration in plasma lipids of HD patients is concomitant with enhanced lipidperoxidation and deficient Vitamin E concentrations and decreased PON-1 activity. Our results, at least partially, might explain advantageous effect of PUFA (fish oil) supplementation which balances PUFA deficiency, and show that supplementation of diet with Vitamin E must be necessary in HD patients.

4.4. Intracellular free calcium levels in neutrophils of HD patients

It was found that in those HD patients who did not receive rHuEpo therapy $[Ca^{2+}]_i$ levels in resting neutrophils is almost double of normal levels (224±323 vs. 105±15 nM, $p<0.01$). After the initiation of rHuEpo therapy 8-12 weeks necessary for normalization of $[Ca^{2+}]_i$. Long-term treatment of HD patients with rHuEpo (>1 y) resulted in normal levels of $[Ca^{2+}]_i$ in almost all patients. When anemia and disturbed calcium-phosphate metabolism of HD patients were taken into consideration we have found, that both parameters influence $[Ca^{2+}]_i$ in resting neutrophils. In those patients who had hgb>100 g/L or htc>0.3 $[Ca^{2+}]_i$ levels in neutrophils were lower (98±15 nM) than that were in others (138±42 nM). When serum calcium was low (<2.1 mmol/L) $[Ca^{2+}]_i$ was elevated (132±48 vs.92±16 nM), and when serum phosphate was high (>1.45 mmol/L) $[Ca^{2+}]_i$ was also elevated (122±42 vs. 92±15 nM). Relationship between $[Ca^{2+}]_i$ of neutrophils and hyperparathyroidism was also demonstrated. Significant correlation was found between $[Ca^{2+}]_i$ and concentrations of serum parathormon concentration ($r=0.473$, $p<0.001$). Role of this parameter in influence of $[Ca^{2+}]_i$ in neutrophils was confirmed by the fact that after parathyreoidectomy not only parathormon levels but $[Ca^{2+}]_i$ in neutrophils was also normalized. $[Ca^{2+}]_i$ in neutrohils was altered by biocompatibility of dialysis membrane. After HD treatment $[Ca^{2+}]_i$ in neutrophils increased using either cuprophne (cup) or polysulfon-base (PS) membrane, however the increase was significant only the use of bioincompatible cup membrane. There was no correlation between the age of patients and $[Ca^{2+}]_i$ in neutrophils, however it correlated with time spent in HD treatment.

In conclusion, elevated $[Ca^{2+}]_i$ in cells of HD patients suggest that they are in prestimulated state, which might be in connection with anemia, disturbances in calcium-phosphate metabolism, HD process etc. rHuEpo treatment has advantageous effect not only on anemia but on $[Ca^{2+}]_i$ in neutrophils as well.

4.5. Hyperhomocysteinaemia in HD patients.

It was demonstrated here, that long-term and low dose of folic acid treatment (3 mg/day) normalized Hcy levels in case of some but not all HD patients. It was also demonstrated that in spite of folic acid treatment, with increasing of Hcy concentrations both folic acid (12±6.2 vs. 21.6±20.2 ug/L) and Vitamin B_{12} (405±352 vs. 1160±582 ng/ml) concentrations decreased in plasma of HD patients, and independently from polymorphism of MTHFR. Though in those HD patients who had elevated Hcy concentrations homozygous form of MTHFR is more frequent than in patient with normal Hcy concentrations (22 vs. 9,2%).

Correlations between serum concentrations of Hcy, cobalt, nickel, Vitamin B_{12} and folic acid were found. In those patients who had elevated serum nickel concentrations (> 5 ug/L) had lower Hcy (17±7.5 vs. 22.2±14.5 umol/L, $p<0.04$) and higher Vitamin B_{12} concentration (623±242 vs. 480±302 ng/ml) than that were in others, and independently from polymorphism of MTHFR.

5. New findings

1. Alterations of both specific and scavenger LDL receptor expressions and functions have been demonstrated in the same experiments.
2. Changes in fatty acid composition of plasma lipids depend on progression of CRF, on severity of hyperlipoproteinaemia, and on cardiovascular complications. Alterations in fatty acids might be in connection with enhanced lipidperoxidation (and vica versa) in HD patients.
3. Intracellular free calcium concentrations in neutrophils of HD patients might be modified by calcium-phosphate metabolism, dialysis time etc. rHuEpo treatment is advantageous not only for treatment of aneemia, but in normalization of $[Ca^{2+}]_i$, as well.
4. HHC might be in connection with disturbed trace element concentrations.

6. Consequences

Those relationships which were found between studied parameters, such as fatty acid composition, Vitamin E concentrations, parameters influencing $[Ca^{2+}]_i$ in neutrophils, trace elements (cobalt and nickel), might have therapeutic usefulness. Normalization all of these parameters might help in decrease of risk factors of atherosclerosis and infections, and progression of renal disease.

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