THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

HEMOLYSIS, HEMOGLOBIN-OXIDATION AND HEME-MEDIATED LIPIDOXIDATION IN ATHEROSCLEROTIC LESIONS

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1. INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) in the subendothelial space is one of the early events in atherogenesis. Oxidized LDL (oxLDL) is readily ingested by macrophages through the scavenger receptor, it is chemotactic for the circulating monocytes and increases monocyte adhesion. OxLDL inhibits the motility of macrophages already present in the lesion, and stimulates release of cytokines and growth factors. *In vitro* oxidation of LDL can be carried out by iron or copper, by metalloproteins, by enzymes (myeloperoxidase, lipooxygenase), and by some vascular cells. We previously suggested that heme, an ubiquitous iron-containing compound mediates the oxidative modification of low-density lipoprotein which modification can be amplified by trace amounts of hydrogen peroxide or polymorphonuclear leukocyte-derived oxidants. Oxidized LDL is not a single, well-defined entity, but has structural, physical and biological properties, which vary according to the degree of oxidation. Free hemoglobin (Hb) in plasma, when oxidized, can provide heme. Cell-free hemoglobin is more readily oxidized to ferrihemoglobin by oxidants such as hydrogen peroxide produced by activated polymorphonuclear cells. In addition to ferrihemoglobin this reaction generates ferrylhemoglobin (FeIII/FeIV=O), an unstable oxidized form of hemoglobin. This form rapidly returns to the ferric (Fe III) state through electron transfer from the globin moiety generating globin radicals. Tyrosyl radicals, for example, can react with each other intramolecularly to produce dityrosine or intermolecularly to form hemoglobin multimers. Oxidized LDL is cytotoxic to vascular endothelium; in response, cells upregulate heme oxygenase-1 (HO-1) and ferritin as a defence against such toxicity. HO is a heme-degrading enzyme that opens the porphyrin ring, producing biliverdin and carbon monoxide and releasing iron. Three genes encode for 3 isoenzymes for HO. HO-1, identified as a 32.8-kD stress protein, is transcriptionally inducible by a variety of agents,

such as heme, oxidants, and cytokines. Heme oxygenase-2 is constitutively active as a metabolizer of heme. Bilirubin may be an important antioxidant and the carbon monoxide produced during the degradation of heme is a regulator of vascular tone and hemostasis. Up-regulation of HO-1 and ferritin in endothelial cells has been reported in the early phase of progression of atherosclerotic lesions. Expression of HO-1 provides protection against atherosclerosis in several experimental models. It inhibits cytotoxicity induced by oxidized LDL in endothelial cells and atherosclerotic lesion formation in LDL-receptor knockout mice whereas inhibition of HO-1 enzyme activity by tin protoporphyrin leads to accelerated atherosclerosis in these mice. Overexpression of HO-1 using an adenoviral HO-1 vector inhibited the development of atherosclerosis in apolipoprotein E-deficient mice. The central importance of HO-1 in vascular biology was highlighted by the discovery of a child with HO-1 deficiency. In the HO-1-deficient patient, both intravascular hemolysis and endothelial cell injury were revealed. Fatty streaks and fibrous plaques in the aorta were reported, as well as mesangioproliferative glomerular changes in the kidney. Sequence analysis of the patient's HO-1 gene showed complete loss of exon 2 of the maternal allele and a 2-nucleotide deletion in exon 3 of the paternal allele. Spectral analysis of the child's plasma revealed a substantial oxidation of hemoglobin leading to heme transfer and heme-iron accumulation in his low-density lipoprotein followed by a subsequent heme-catalyzed oxidation of LDL, as it was described by our laboratory.

Morbidity and mortality from atherosclerosis is largely due to the rupture of advanced atheromatous lesions (types IV and Va according to Stary), which are characterized by extensive accumulation of extracellular lipid localized in the lipid core and thin fibrous cap. Although hematomas may be caused by fissures at the lesion surface accompanied by hematoma/hemorrhage and thrombus formation, there is evidence that some hematomas may begin within the lesions as hemorrhages from neovasculature that sprout from the vasa vasorum.

Oxysterols and oxidation products of polyunsaturated fatty acids are present in human atheromatous lesions that are hazardous regions for nucleated cells (both endothelial cells and macrophages). The major cytotoxic species are lipid hydroperoxides (LOOHs), aldehydes and carbonyls.

2. AIMS OF THE STUDY

1. We planned to describe the association between the degree of heme-mediated oxidative modification of LDL and its biological properties. We tested whether reducing agent H_2S might inhibit hemin-mediated oxidative modification of LDL.
2. We tried to describe the possible reactions between oxidized LDL, heme and hemoglobin, to better understand the pathophysiology beyond severe atherosclerosis in the case of HO-1 deficiency. We hypothesized that LDL-associated lipid hydroperoxides could oxidize hemoglobin.
3. We tested the hypothesis that heme-iron may accumulate in atherosclerotic lesions by intrusion and lysis of erythrocytes. Liberated hemoglobin is oxidized, and released heme-iron dependent oxidation of lipids is strongly favored, contributing to further plaque development.
4. We wondered if cells of the HO-1-deficient patient were prone to oxidative damage arising from heme-mediated oxidation of LDL

3. METHODS

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were removed from human umbilical veins and cultured in modified medium 199. Human HO-1 deficient and control immortalized lymphocyte cell lines (LCLs) were generous

gifts from Akihiro Yachie (Kanazawa University, Japan) and were cultured in RPMI 1640.

Preparation and oxidation of low density lipoprotein

LDL was isolated from plasma derived from EDTA-anticoagulated venous blood of healthy volunteers by gradient ultracentrifugation.

Measurement of lipid peroxidation parameters in LDL

Concentrations of conjugated diene and thiobarbituric acid-reactive substances (TBARs) were determined by spectrophotometry at 234 nm and 532 nm, respectively. **F**errous **O**xidation of **X**ylenol orange method was used to measure the total lipid hydroperoxides.

Tissue samples

Specimens of human atherosclerotic lesions were obtained from aorta or its primary branches of beating-heart donors for organ transplantation. For histopathological examination hematoxilin-eosin staining was performed.

Measurement of lipid peroxidation parameters and heme in tissue samples

Lipids of blood vessel samples were extracted from tissue by chloroform-methanol (2:1 v/v). Conjugated diene content, total lipid hydroperoxides, TBARs, iron and heme were determined spectrophotometrically.

Determination of neutral lipids and fatty acid

Neutral lipids were determined by thin layer chromatography. For fatty acid analyses, the lipid extracts were hydrolyzed and methylated. Fatty acid methylesters were examined by gas chromatography. α-tocopherol content was measured by high performance liquid chromatography.

Determination of tissue iron content

Iron in tissue was determined spectrophotometrically as a ferrozine-iron complex in a reducing environment.

Hemoglobin preparation

Purified ferrohemoglobin was prepared from lysed fresh human red cells using ion-exchange chromatography.

Preparation of human neutrophils

Polymorphonuclear leukocytes (PMNs) were isolated from heparin-anticoagulated venous blood of healthy volunteers by multistep centrifugation.

Cell cytotoxicity assay

Viability of endothelial cells or immortalized lymphocyte cells were determined by MTT-reduction ((3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium-bromide).

Real time RT-PCR to determine HO-1 mRNA

Total RNA was isolated from cells using the RNAzol STAT-60 (TEL-TEST Inc., Friendswood, TX). HO-1 and cyclophilin (housekeeping gene) levels were measured by real time PCR using fluorescent TaqMan probes following reverse transcription.

Western blot for HO-1 protein

Protein was subjected to SDS-PAGE (12.5%) and transferred to a nitrocellulose membrane. HO-1 was identified using a polyclonal antibody. Antigen-antibody complex was visualized with a horseradish peroxidase chemiluminescence system. For loading control membranes were re-probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody.

HO enzyme activity assay

HO activity in endothelial cell microsomes was measured by bilirubin formation after treating the cells grown in Petri dishes.

Measurement of hemoglobin oxidation markers

Protein oxidation marker, dityrosine was detected using high performance liquid chromatography. Crosslinked hemoglobin was examined by Western blot analysis (12.5% SDS-PA gel).

4. RESULTS AND DISCUSSION

4.1. HEME-MEDIATED OXIDATION OF LDL

4.1.1. Oxidation of LDL with heme and hydrogen peroxide

LDL oxidized with heme and hydrogen peroxide was found to be markedly cytotoxic to endothelial cells, however incubation of the mixture at 37°C for 24 hours or more prior to addition to the cells decreased cytotoxicity substantially. Similarly to cytotoxicity HO-1 expression was also dependent on the time of LDL oxidation. We measured the concentration of lipid peroxidation products on the same time scale and found that both cytotoxicity and expression of HO-1 in endothelium strongly correlated to the lipid hydroperoxide content of oxidized LDL.

4.2.2. Oxidation of LDL with heme alone

Heme alone moderately oxidizes LDL in 6-12 hours. Repeated heme exposure, however, increases the LOOH content and the toxicity of the LDL within minutes. LDL from the HO-1 deficient patient was mildly oxidized and slightly cytotoxic. After heme exposure, however, a rapid increase in LOOH content of lipoprotein and a subsequent marked endothelial cytolysis was observed, similarly to that of moderately oxidized LDL from healthy subjects.

4.1.3. Effect of hydrogen sulfide on LDL-oxidation

Hydrogen sulfide (H_2S) dose dependently delayed the accumulation of lipid peroxidation products during hemin-mediated oxidation of LDL. Moreover, H_2S decreased the LOOH content of oxidized LDL which was accompanied by reduced cytotoxicity and moderated induction of heme oxygenase-1. H_2S directly protected endothelium against hydrogen peroxide and oxLDL-mediated cytotoxicity.

4.2. HEMOGLOBIN OXIDATION

Ferrohemoglobin can readily be oxidized to heme-releasing ferrihemoglobin in the presence of inflammatory cell-derived oxidants. Oxidation of ferrohemoglobin by activated polymorphonuclear leukocytes (PMNs) occurred and was inhibited by catalase, indicating that this conversion was mediated by hydrogen peroxide. We raised the question whether lipid hydroperoxide associated with LDL could oxidize ferrohemoglobin. We demonstrated that oxidatively modified LDL converted ferrohemoglobin to ferrihemoglobin *in vitro.* The kinetics and the degree of oxidation strongly depended on the concentration of LDL-associated lipid hydroperoxides. Reduction of lipid hydroperoxide content of LDL with GSH peroxidase reduced the formation of ferrihemoglobin.

4.3. HEME AND HEMOGLOBIN-MEDIATED LIPID PEROXIDATION IN ATHEROSCLEROTIC LESIONS

4.3.1. Classification of atherosclerotic lesions involved the study

Blood vessel sections were classified by their morphology into three groups: controls, atheromatous lesions (atheromas) and complicated lesions. Products of lipid peroxidation, iron, α-tocopherol, lipid and hemoglobin contents of tissue samples were measured and lipid and fatty acid composition were determined. Results show that the extent of lipid oxidation increases with the development of the lesion.

4.3.2. Interaction of lesion lipids and red blood cells

Lipids in atherosclerotic plaques can contact with red blood cells when plaque fissure occurs or when hemorrhage is generated from unstable neovasculature sprouting from the vasa vasorum. Atheroma lipids caused significant lysis of red

cells and cell-free hemoglobin underwent oxidation. Preincubation of lipid extracts with glutathione/glutathione peroxidase (which specifically reduced lipid hydroperoxide to alcohol) significantly lowered the lytic effect supporting the assumption that LOOHs have crucial role in hemolysis and oxidation of hemoglobin.

4.3.3. Interaction of lesion lipids and hemoglobin.

Both ferro- and ferrihemoglobin induced the oxidative modification of atheromatous lesion lipids. Since oxidatively modified LDL was shown to oxidize ferrohemoglobin we assessed whether lipid hydroperoxides in the lipid extracts of atheromatous lesion might also oxidize ferrohemoglobin. Indeed, lipid derived from atheromatous lesions promoted the oxidation of ferrohemoglobin to ferrihemoglobin, meanwhile lipid itself also became more oxidized.

Hemoglobin extracted from complicated lesions was mainly oxidized (50 % ferrihemoglobin). Moreover, we detected dityrosine and hemoglobin multimers, in hemoglobin of complicated lesions. These protein oxidation markers are hallmarks of formation of ferrylhemoglobin (FeIII/FeIV=O), a highly unstable oxidized form of hemoglobin generated in hydroperoxide-mediated oxidation of hemoglobin.

4.3.4. Reaction of lesion lipids and heme

Heme induces peroxidation of lipid extract derived from atheromatous lesions; in parallel heme degradation occurs.

4.3.5. Reaction of oxidized atheroma lipids with endothelial cells

Lipids from atherosclerotic lesions were cytotoxic to endothelium, an effect strikingly enhanced when lipids were pre-oxidized by exposure to heme. At sublethal doses, atheroma lipid induced the expression of the stress-responsive gene HO-1, at both mRNA and protein levels. In contrast, lipids from control

blood vessels were not cytotoxic and failed to affect the expression of HO-1 in endothelium.

4.3.6. Inhibition of lipid oxidation

The chemical changes exerted by heme on lipids isolated from atheromatous lesions were attenuated by antioxidants such as BHT, α-tocopherol, the iron chelator deferoxamine, and the heme binding protein, hemopexin. Hemoglobin-mediated oxidative modification of lipid extracted from atheromatous lesions was inhibited by haptoglobin via stabilizing the binding of heme to globin and by hemopexin, indicating the necessity of heme release from ferrihemoglobin for oxidative process. Inhibition of lipid oxidation reduced both cytotoxicity and induction of HO-1 expression at the mRNA and protein levels.

We suggested a model for red cell-mediated progression of atherogenesis, involving: 1) Red blood cells infiltrate the atheromatous lesion; 2) Erythrocyte lysis, liberation and oxidation of ferrohemoglobin occurs producing ferri- and ferrylhemoglobin 3) Heme is released from ferrihemoglobin; 4) Heme uptake by atheroma lipid; 5) Amplification of lipid oxidation in atheroma; 6) Damage of endothelium and induction of heme oxygenase-1 and ferritin by atheroma lipid and heme.

4.4. HO-1 DEFICIENT CELLS AND OXIDIZED LIPIDS

Immortalized lymphocyte cell line derived from the HO-1- deficient patient was demonstrated to have increased sensitivity to heme toxicity. We found that oxidized LDL and heme-treated atheroma lipids were more cytotoxic to the HO-1-deficient cells than to the control cells, suggesting that HO-1 is an important element of the defense mechanism. At sublethal doses, oxLDL induced HO-1 mRNA in both cell lines suggesting that promoter region of the gene is not affected by the mutations in the HO-1-deficient patient. In contrast, heme and

oxidized LDL increased HO-1 enzyme activity in the wild type cells with no significant increase in the HO-1 enzyme activity in the HO-1-deficient cells.

5. SUMMARY

● Cytotoxicity and expression of HO-1 in endothelium caused by heme-treated LDL are strongly correlated to the lipid hydroperoxide content of oxidized LDL.

● LDL of the HO-1 deficient patient was mildly oxidized slightly cytotoxic. However, it was hazardous as heme exposure substantially increases the LOOH content and the toxicity of the LDL within minutes.

● Hydrogen sulfide (H_2S) delays the heme-mediated oxidation of LDL.

● H_2S decreased the LOOH content of oxidized LDL which was accompanied by reduced cytotoxicity and moderated induction of heme oxygenase-1.

● H_2S directly protected endothelium against hydrogen peroxide and oxLDL-mediated cytotoxicity.

● Lipid hydroperoxides associated with LDL can dose-dependently oxidize ferrohemoglobin, as it happened in the plasma of the HO-1 deficient patient.

● Levels of lipid peroxidation parameters and iron were higher in complicated lesions thant in atheromas indicating that oxidation of lipids in lesions is intesified with plaque development.

● Atheroma lipids caused significant lysis of red cells and cell-free hemoglobin underwent oxidation.

● Both ferro- and ferrihemoglobin induced the oxidative modification of atheromatous lesion lipids.

● Hemoglobin from complicated lesions were mainly oxidized and contained protein oxidation markers, dityrosine and hemoglobin multimers, hallmarks of

formation of ferrylhemoglobin (FeIII/FeIV=O), a highly unstable oxidized form of hemoglobin generated in hydroperoxide-mediated oxidation of hemoglobin.

● Heme induces peroxidation of lipid extract derived from atheromatous lesions.

● Oxidized lipids from atherosclerotic lesions were cytotoxic to endothelium and at sublethal doses, induced the expression of the stress-responsive gene HO-1, at both mRNA and protein levels.

● Lipid oxidation was attenuated by antioxidants (BHT, α-tocopherol), the iron chelator deferoxamine, and the heme binding protein, hemopexin and hemoglobin-complexing haptoglobin. Inhibition of lipid oxidation reduced endothelial cell reactions as well.

● Oxidized LDL and heme-treated atheroma lipids were more cytotoxic to the HO-1-deficient cells than to the control immortalized lymphocyte cells.

● At sublethal doses, heme and oxidized LDL induced HO-1 mRNA in both cell lines, however they increased HO-1 enzyme activity in the wild type cells with no significant increase in the HO-1-deficient cells.

6. LIST OF ORIGINAL PUBLICATIONS

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