Plasma oxidized lipoprotein(a) and its immune complexes are present in newborns and children

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A B S T R A C T

Background: Oxidized Lp(a) [ox-Lp(a)] has been reported to play more potent roles than native Lp(a) in atherosclerosis. We investigated the distribution characteristics of plasma ox-Lp(a) and Lp(a) immune complex [Lp(a)-IC] levels in newborns and children.

Methods: Plasma ox-Lp(a) and Lp(a)-IC levels were measured in 747 children and 30 cord blood by ELISAs.

Results: The mean levels of Lp(a), ox-Lp(a) and Lp(a)-IC were much lower in newborns than in children (P<0.001), and increased rapidly to that in children after birth. The distributions of Lp(a), ox-Lp(a) and Lp(a)-IC were skewed toward low values in children, no difference of their levels was found in each of the 13 year groups. The levels of ox-Lp(a) correlated positively with total and LDL cholesterol, Lp(a) and Lp(a)-IC; Lp(a)-IC correlated positively with sex, total and LDL cholesterol, Lp(a) and ox-Lp(a), respectively. Multiple linear regression analysis showed Lp(a) and Lp(a)-IC accounted for 42% of the variation in ox-Lp(a) levels, and ox-Lp(a) accounted for 30% of that in Lp(a)-IC.

Conclusions: The fact that ox-Lp(a) and Lp(a)-IC are present in newborns and children suggests that oxidized lipoproteins play an initiating role in atherosclerotic process.

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Abbreviations: Lp(a), lipoprotein(a); LDL, low density lipoprotein; apo, apolipoprotein; ox-Lp(a), oxidized Lp(a); IC, immune complex; ox-LDL, oxidized LDL; SR-A, scavenger receptor A.

1. Introduction

Lipoprotein(a) [Lp(a)] is an atherogenic particle that structurally resembles a low density lipoprotein (LDL) particle but contains a molecule of apolipoprotein(a) [apo(a)] attached to apoB-100 by a disulfide bond [1], increased plasma concentrations of Lp(a) have been shown to be one of the independent risk factor for atherosclerosis [2–4]. Oxidized Lp(a) [ox-Lp(a)] has been reported to play a more potent role than native Lp(a) in atherosclerosis. Ox-Lp(a) is a ligand for scavenger receptors [5,6] and might reasonably be expected to contribute to foam cell formation. Ox-Lp(a) may also induce adhesion molecule expression on monocytes, promoting their recruitment and adhesion to the endothelium [7], and modified forms of Lp(a), some resembling ox-Lp(a), have been identified in human atheromatous lesions [8]. In addition, in vivo oxidative modification increases the inhibitory effect of Lp(a) on plasminogen binding to cell surfaces, which could attenuate fibrinolytic activity by reducing plasminogen activation [9]. Ox-Lp(a) [10–12], its autoantibodies [13], and Lp(a) immune complexes [Lp(a)-IC] have all been detected in vivo [14,15]. Interestingly, our previous studies have shown that ox-Lp(a) and its IC concentrations increase in patients with coronary artery disease (CAD) and in rheumatoid arthritis patients with excessive cardiovascular events [10,14,16].

Several lines of evidence support the concept that oxidized LDL (ox-LDL) plays a pivotal role in the development of atherosclerosis. Ox-LDL levels have been reported to be a useful marker for identifying patients with CAD and to be positively related with the severity of acute coronary syndromes (ACS) [17–22]. Though it has been reported that human plasma contains small but detectable amount of oxidized lipoproteins, several important questions still remain to be answered, including the source of plasma oxidized lipoproteins, and an initiating or a contributing role of oxidized lipoproteins playing in the development of atherosclerosis. It has also been shown that atherosclerosis begins in childhood, and early arterial lesions develop early in life at least 20 years before the onset of the clinical symptoms [23]. Therefore, it is extremely important to investigate the distribution characteristics of plasma oxidized lipoproteins in children as well as in newborns. However, to our knowledge, no study to date has evaluated whether ox-Lp(a) and Lp(a)-IC are present in children and newborns. So we detected the plasma ox-Lp(a) and Lp(a)-IC levels in healthy children with different age and newborns, and investigated their possible associations with plasma lipid parameters, age, and sex in this study.

2. Materials and methods

2.1. Study subjects

Seven hundred and forty-seven healthy children (261 girls and 486 boys), who had visited Nanjing Children’s Hospital for a routine check-up, were enrolled. Their age...
range was from 1 month to 15 years. Thirty cord bloods were also collected from healthy newborns (14 female and 16 male) with gestational ages between 39 and 41 weeks. All the children were randomly selected by routine healthy examination, including physical and electrocardiographic examinations, and laboratory tests. Exclusion criteria were coronary artery disease, myocardial infarction, diabetes mellitus, hypertension, liver or kidney disease, hyperlipidemia, lipid-lowering therapy or malignant disease. The subjects were not on any medications and did not have any other definitive diseases. All the newborns were appropriately grown during the period of fetuses. The subjects were living in Nanjing of China, Han population, which is the major ethnic group in China. The bloods from children were sampled 10 h after fasting and the bloods from the neonate were 2 h, then they were collected into EDTA (1 mg/ml) containing tube and plasma were separated immediately and stored at −70 °C until analysis. The study protocol had been approved by the ethical committees of hospitals, and informed consent was obtained from the parents of all the children and newborn.

2.2. Enzyme-linked immunosorbent assays (ELISA) for ox-Lp(a) and Lp(a)-IC

Ox-Lp(a) was measured by a sandwich ELISA, using polyclonal antibody against ox-LDL as the capture antibody and quantitating with monoclonal anti-apo(a) enzyme conjugate as previously described [10]. Antibodies to ox-LDL were obtained by immunization of New Zealand white female rabbits with ox-LDL. The resulting rabbit antisera was first fractionated by affinity chromatography with immobilized protein G, and the IgG fractions were then absorbed in a column of immobilized native LDL. The washout from the column of immobilized native LDL, containing antibodies to ox-LDL and irrelevant IgG, was used to capture ox-LDL, which had no reactivity with native LDL. A pooled fresh-frozen plasma sample (mixed plasma from 50 healthy subjects) was used as reference plasma of ox-Lp(a). The value of ox-Lp(a) was determined by the ELISA repeatedly, based on the concentration of copper ion oxidized Lp(a) as the standard.

Lp(a)-IC was measured by a sandwich ELISA, using anti-human IgG as the capture antibody and quantitating with monoclonal anti-apo(a) enzyme conjugate as previously described [14]. A pooled fresh-frozen plasma sample (from 50 healthy subjects) was used as reference plasma of Lp(a)-IC and the value was expressed as 1 relative absorbance unit (AU).

2.3. Lp(a) and lipids concentration determination

Lp(a) level was detected by a "sandwich" ELISA as previously described [24]. With the use of monoclonal anti-apo(a) as capture antibody, bounded Lp(a) particles were quantitated with polyclonal anti-apo(a) enzyme conjugates. Polyclonal sheep anti-apo(a) (a) was self-made. Cross-reactivity in these Lp(a) antisera against LDL, plasminogen and other apolipoproteins was removed by absorbing on an affinity chromatographic column of Sepharose 4B (Pharmacia) coupled with plasminogen and pooled sera obtained from Lp(a)-negative subjects. Reference serum of Lp(a) was from Immuno AG, Vienna. Total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) cholesterol and LDL cholesterol (Daiichi Pure Chemicals, Japan) were measured on a Hitachi 7600 analyzer.

2.4. Statistical analysis

Statistical analyses were performed with SPSS 11.5. The values were expressed as mean ± SD. The distributions of Lp(a), ox-Lp(a), and Lp(a)-IC levels were skewed and were log transformed to normalize their distribution. The differences between variants were analyzed by Student’s t-test or ANOVA, and the differences between groups were subsequently determined by Fisher LSD test. Correlations between variables were calculated by the non-parametric Spearman rank coefficient test. A multiple regression test was performed, considering the levels of ox-Lp(a) or Lp(a)-IC levels as dependent variables, with the independent variables consisting of sex, age, the levels of lipid parameters, Lp(a), and Lp(a)-IC or ox-Lp(a). A P < 0.05 was considered statistically significant.

3. Results

3.1. Frequency distribution of ox-Lp(a), Lp(a)-IC concentrations in newborns and children

The distributions of Lp(a), ox-Lp(a), and Lp(a)-IC levels were all skewed toward lower values in the 747 children (Fig. 1). The distribution ranges of Lp(a), ox-Lp(a), and Lp(a)-IC levels in 30 newborns were narrow (12.0–89.00 mg/L, 1.54–2.20 mg/L, and 0.13–0.17 AU, respectively); while their ranges were very wide in the children (9.40–913.58 mg/L, 0.60–72.00 mg/L, and 0.01–12.5 AU, respectively).

3.2. Mean ox-Lp(a), Lp(a)-IC concentrations in healthy newborns and in children with different age and sex

Table 1 shows the differences of the lipid parameters and levels of Lp(a), ox-Lp(a) and Lp(a)-IC in the newborns and children according to sex. Lp(a), ox-Lp(a), Lp(a)-IC levels, and lipid parameters were all markedly lower in the newborns than in children (P < 0.001). Lp(a), ox-Lp(a) and Lp(a)-IC levels of the newborns were 21, 8, and 13 percent of the children’s, respectively. In the newborns, no difference of the above variables was found between males and females. In the children, Lp(a)-IC and TG levels in the females were higher than in the males. Mean levels of Lp(a), ox-Lp(a) and Lp(a)-IC in each of the
13 year groups were also studied, while no difference of their levels was found among the age groups (Fig. 2).

3.3. Relationship between ox-Lp(a), Lp(a)-IC, Lp(a) level, sex, age, and lipid variables

Table 2 gives Spearman rank correlations between sex, age, lipid parameters, Lp(a), ox-Lp(a), and Lp(a)-IC in the children. The levels of ox-Lp(a) correlated positively with TC, LDL cholesterol, Lp(a) and ox-Lp(a). We next performed the multiple linear regression analysis for ox-Lp(a) or Lp(a)-IC versus each factor, respectively; a backward elimination procedure of stepwise analysis was used. Consequently, Lp(a) and Lp(a)-IC accounted for 42% of the variation in ox-Lp(a), and only ox-Lp(a) accounted for 30% of the variation in Lp(a)-IC levels (Table 3).

4. Discussion

The mean levels of Lp(a), ox-Lp(a) and Lp(a)-IC in the newborns were very low, and increased rapidly to that in children after birth. Furthermore, no difference of their levels was found in each of the 13 year groups in the children.

Ox-Lp(a) has been reported to play more potent roles than native Lp(a) in atherosclerosis and to be detectable in blood. It has also been accepted that not only LDL, but also Lp(a) triggers an immune response leading to the production of autoantibodies and subsequently to the formation of IC. Theoretically, apoB and apo(a) proteins of Lp(a) molecules can both be oxidatively modified in vivo. The degree of oxidized apo(a) or apoB protein of Lp(a) has been detected to estimate circulating ox-Lp(a) levels [11,12]. We previously have also developed 2 ELISAs for measuring plasma ox-Lp(a) level, using human autoantibodies against ox-Lp(a) or specific rabbit antiserum against human ox-LDL as the capture antibody and quantitating with monoclonal anti-apo(a) enzyme conjugate, respectively. For the autoantibodies isolated from human mixed plasma can recognize both apo(a) and apoB epitopes of ox-Lp(a), the developed ELISA for ox-Lp(a) by using autoantibody may more accurately reflect the state of Lp(a) oxidation in vivo. Furthermore, a significantly positive relationship was found between ox-Lp(a) levels detected by the two ELISAs [10]. Ox-LDL can't be detected by the ELISAs, because of using monoclonal antibody against apo(a) as quantitating antibodies. In addition, for plasma ox-LDL levels are low and samples are diluted, plasma ox-LDL has no influence on the assays.
oxidized apoB protein of Lp(a) and Lp(a)-IC levels in the children and newborn were explored. Our results showed that cord blood contained small but detectable amount of Lp(a), ox-Lp(a) and Lp(a)-IC. The Lp(a) level in newborn was approximately one-fifth of that in the children, which was similar to what Abe et al. reported [25]. After birth, Lp(a) is assumed to be produced rapidly by the liver and the level in plasma increases to the adult level. LDL cholesterol or apoB protein levels have been reported to be positively related with ox-LDL and its IC levels [26]. Similarly, the associations among plasma Lp(a), ox-Lp(a) and Lp(a)-IC levels were also found in the study. Furthermore, the multiple regression analysis showed that Lp(a) and Lp(a)-IC accounted for 30% of the variation in ox-Lp(a), and ox-Lp(a) accounted for 42% of the variation in Lp(a)-IC levels, which may be explained by the fact that high levels of Lp(a) mean more Lp(a) have chance to be oxidized in vivo, and resulting in strong immune response and leading to the formation of Lp(a)-IC. Lp(a) level is mainly genetically determined, and doesn’t correlate with age or sex and is insensitive to dietary and environmental factors [1]. Though our data showed that Lp(a)-IC levels in the female children were higher than in the males, the mean levels of ox-Lp(a), Lp(a)-IC, and Lp(a) remained unchanged in each of the 13 year groups children. Tinahones et al. [27] reported sex had no influence on levels of LDL-IC in the general population, while LDL-IC levels were significantly higher in younger persons than in older persons, which may be caused by the fact that plasma LDL level is sensitive to age, dietary and environmental factors. Islam et al. [28] reported that small apo(a) phenotypes had a potential influence on the levels of LDL-IC in children. Further studies are needed to evaluate the effect of the presence of higher levels of ox-Lp(a) and Lp(a)-IC, as well as the degree of oxidized lipoprotein in predicting early atherosclerosis.

Oxidized lipoprotein results from exposure of lipoprotein to oxidizing species, such as superoxide anion and hydrogen peroxide derived from all cells present in the artery wall, particularly macrophages, as well as enzymes such as lipoxygenases and products of myeloperoxidase, resulting in oxidation of the lipid and protein components [29]. The source of plasma oxidized lipoprotein isn’t clear. It has been reported that ox-LDL does not originate from extensive metal ion-induced oxidation of LDL but that it might be generated by cell-associated oxidative enzymatic activity in the arterial wall [30]. It has also been demonstrated in animal models that the oxidation of LDL indeed occurs in the arterial wall and not in the blood [31,32]. Furthermore, ox-LDL has been reported to strongly reflect the presence of ACS. Oxidized phospholipid has also been found preferentially associated with Lp(a) [33] and to be correlated with both the presence and extent of angiographically documented CAD [34,35], and their levels increase after ACS [21] and immediately after percutaneous coronary intervention [36]. Similarly, our unpublished data shows that elevated plasma levels of ox-Lp(a) and the ratio of ox-Lp(a) to Lp(a) also reflect the presence and extent of angiographically documented CAD, especially clinically expressed in ACS. Overall, these studies make a strong argument that increased oxidized lipoproteins in the blood in CAD mainly come from the arterial wall, such as directly released from ruptured or permeable plaques. In addition, it has been reported that antibodies against ox-LDL and LDL-IC are easily detectable in asymptomatic young adults and children [27,28,37]. The present study also show that ox-Lp(a) and Lp(a)-IC have been detected in all the healthy children with different age and sex, especially in newborns, for early arterial lesions may not present in all the children, which suggests plasma oxidized lipoproteins in normal subjects come from other pathways, except from ruptured or permeable plaques. It is noteworthy that LDL isolated from normal intima already shows decreased contents of polyunsaturated fatty acids, which may indicate limited oxidation of these particles [38]. The limited oxidized lipoproteins in normal intima may flow back into blood. It is also possible that oxidation-specific epitopes generated in the arterial intima, such as oxidized phospholipids, might have been transported by some mechanisms to lipoproteins acceptors. It is also likely that oxidation of lipoproteins occurs partly in blood to a minor extent.

Oxidized lipoprotein in the arterial intima is taken up by macrophages via scavenger receptor A (SR-A) type I and type II [39], promoting foam cell formation and fatty streak development. In the clearance of oxidized lipoprotein from circulating, such as ox-LDL, SR-A appears to have much less importance, while most of the hepatic uptake of ox-LDL is attribute to Kupffer cell that normally possesses SR-A and other candidate receptors for ox-LDL such as Fcg receptor (FcγR) that can bind Fe portion of immunoglobulin G (IgG) of LDL-IC [40,41]. Thus, in vivo oxidized lipoprotein is cleared by the above mentioned different approaches; meanwhile, the new sources of oxidized lipoprotein enter the circulation again. This remains dynamic balance of circulating oxidized lipoprotein levels in normal subjects.

The above balance and similar oxidative stresses may be the reasons that the ratios of ox-Lp(a) and Lp(a)-IC to Lp(a) and their levels remain similar in the studied children and our previously reported healthy adult [10,14].

One of important problems remaining to be answered is whether the response to oxidized lipoprotein plays an initiating or a contributing role in the development of atherosclerosis. To answer this question through clinical studies, one major problem is the difficulty encountered in defining atherosclerosis-free individuals. The results that high levels of anti-ox-LDL antibodies and LDL-IC are present in individuals with confirmed atherosclerosis suggest that the immune response to ox-LDL may be a secondary phenomenon, possible contributing to the development of atherosclerosis. However, the present and other studies have shown that ox-Lp(a) and Lp(a)-IC are present in all the healthy children and especially in newborns, and that anti-ox-LDL antibodies and LDL-IC are easily detectable in asymptomatic young adults and children [27,28,37], which suggest that the immune response to modified lipoproteins could appear very early in the process, perhaps being one of the initiating factors. In fact, oxidative stress has been reported to be present early in pregnancy and children, and to be associated with arterial dysfunction and enhanced intima-media thickness [42,43]. In general, it has been accepted that lipoprotein traverses the subendothelial space where it becomes oxidized, and may induce endothelial dysfunction, one of the earliest manifestations of atherosclerosis. In vitro and in vivo studies have shown that ox-LDL promotes endothelial cell toxicity and vasoconstriction [44]. The present data suggest that part of lipoproteins have

### Table 2

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been oxidized before they traverse the subendothelial space, prior to advanced lesion formation, and directly participating in the development of atherosclerosis.

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References