The pro-inflammatory effect of uraemia overrules the anti-atherogenic potential of immunization with oxidized LDL in apoE−/− mice

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Abstract

Background. Uraemia increases oxidative stress, plasma titres of antibodies recognizing oxidized low-density lipoprotein (oxLDL) and development of atherosclerosis. Immunization with oxLDL prevents classical, non-uraemic atherosclerosis. We have investigated whether immunization with oxLDL might also prevent uraemia-induced atherosclerosis in apolipoprotein E knockout (apoE−/−) mice.

Methods. ApoE−/− mice were immunized with either native LDL (n = 25), Cu2+-oxidized LDL (n = 25), PBS (n = 25), the apolipoprotein B-derived peptide P45 (apoB-peptide P45) conjugated to bovine serum albumin (BSA) (n = 25) or BSA (n = 25) prior to induction of uraemia by 5/6 nephrectomy (NX).

Results. Immunization with oxLDL increased plasma titres of immunoglobulin G (IgG) recognizing Cu2+-oxLDL and malondialdehyde-modified LDL (MDA-LDL). However, 5/6 NX induced a marked increase in plasma concentrations of anti-oxLDL antibodies as well as pro-atherogenic cytokines [interleukin (IL)-2 (IL-2), IL-4, IL-6 and IL-12] in native mouse LDL (nLDL)-, oxLDL- and PBS-immunized mice. Even though nLDL- and oxLDL-immunized mice displayed higher anti-MDA-LDL IgG titres than the PBS group, aortic atherosclerosis lesion size was

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not affected by immunization. Immunization with the apoB-peptide P45, which consistently reduces classical atherosclerosis in non-uraemic mice, also did not reduce lesion size in uraemic apoE−/− mice.

**Conclusion.** The results suggest that the pro-inflammatory and pro-atherogenic effect of uraemia overrules the anti-atherogenic potential of oxLDL immunization in apoE−/− mice.

**Keywords:** atherosclerosis; oxLDL immunization; uraemia

### Introduction

Chronic renal failure (CRF) causes uraemia, i.e. accumulation of waste products in plasma. Uraemia accelerates development of atherosclerosis and increases the risk of cardiovascular disease (CVD). The risk of CVD is increased up to ~300-fold in young uraemic patients [1–4]. Therefore, it is disappointing that statins have failed to reduce CVD risk in CRF patients [5,6], and there is an urgent need to identify new strategies to prevent CVD in the growing group of patients with CRF.

Several lines of evidence support the idea that oxidative stress may play a major role in uraemic atherosclerosis. CRF patients have elevated lipid peroxidation products in plasma [7,8]. The uraemic 5/6 nephrectomized mouse model (which displays moderate uraemia and markedly accelerated development of atherosclerosis) has increased plasma levels of oxidized low-density lipoprotein (oxLDL) and accumulates oxidatively modified proteins in atherosclerotic lesions [9–11]. Moreover, in this mouse model, uraemia is associated with a marked rise in plasma titres of antibodies reacting with oxidatively modified LDL (oxLDL). It is conceivable that immune mechanisms directed against oxidatively modified lipids may affect uraemic atherogenesis. However, at this stage, it remains controversial whether there is a positive or negative association between plasma titres of antibodies reacting with oxLDL and risk of CVD, and even whether the plasma antibody titres are generally increased or decreased in uraemic patients [12–15].

Despite the controversial predictive role of plasma antibody titres, immunization with oxLDL, malondialdehyde-modified LDL (MDA-LDL) (which increases plasma titres of antibodies reacting with oxLDL) or selected peptides derived from apolipoprotein B (apoB), including the peptide P45 (apoB-peptide P45), has consistently attenuated atherosclerosis in hyperlipidaemic atherosclerosis-prone animal models (previous studies are summarized in Supplementary Table 1). Efforts are currently being made to assess the feasibility of vaccination strategies to prevent CVD in humans. If vaccination approaches also appear to reduce the risk of CVD in humans, patients with mild to moderate uraemia would benefit in particular, since they often progress to end-stage renal disease with a very high risk of CVD. Therefore, in the present study, we explored whether immunization with oxLDL or the apoB peptide P45 might be used to prevent the markedly accelerated development of atherosclerosis in uraemic apolipoprotein E knockout (apoE−/−) mice.

### Materials and methods

**Mice**

Male apoE−/− mice (C57BL/6Jom-ApoEtm1Unc, Taconic M&B Laboratory Animals and Services for Research, Ry, Denmark) were kept on a 12-h light/dark cycle in a temperature-controlled room at 21–23°C with free access to water and standard mouse chow (Altromin 1314, Altromin, Lage, Germany). Each mouse was vaccinated subcutaneously in the right or left inguinal area at the ages of 6, 9 and 11 weeks with (i) PBS, (ii) native mouse LDL (nLDL) (first immunization: 300 μg protein per kilogram, second and third immunization: 600 μg/kg), (iii) oxLDL (first immunization: 300 μg/kg, second and third immunization: 600 μg/kg), (iv) 50 μg of the apoB-peptide P45 conjugated to 50 μg of the carrier: cationized bovine serum albumin (BSA) dissolved in 0.083 M sodium phosphate, 0.9 M NaCl pH 7.2, according to the manufacturer's instruction (catalogue number 77652, Pierce, Rockford, IL, USA) or (v) 50 μg cationized BSA in a total volume of 100 μL. The amino acid sequence of P45 was described by Fredrikson et al. [16]. LDL was prepared as described below.

Moderate uraemia was induced by 5/6 nephrectomy in two operations as previously described [17]. Briefly, at the age of 12 weeks, both poles of the right kidney were removed, and 2 weeks later, the entire left kidney was removed. At the age of 30 weeks, mice were anaesthetized and perfused, and the heart and aortic arch were isolated and prepared as described previously [17]. The experiments were performed according to the principles stated in the Danish law on animal experiments and were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

**Preparation of nLDL and oxLDL**

LDL (1.006 < d <1.063) was isolated from male apoE−/− mouse plasma by sequential ultracentrifugation in the presence of an antioxidant (0.3 mM Na2EDTA). Samples were ultracentrifuged at 50 000 × g, 10°C for 20 h in a Beckman TLA-100 rotor using an Optima Max-E ultracentrifuge (Ramcon, Birkerød, Denmark). The purified LDL was split in two portions. One part (nLDL) was dialysed against PBS with Na2EDTA and kept at ~80°C. The other part (oxLDL) was dialysed against PBS, oxidized with 5 μM CuSO4 for 6 h at 37°C, dialysed against PBS with EDTA and kept at ~80°C. Aliquots of nLDL and oxLDL were examined by agarose gel electrophoresis to ensure the integrity of the lipoprotein preparations and to test the extent of modification of the oxLDL. Just before the immunizations, the adjuvant Injeft alun (Pierce, Rockford, IL, USA) was added to nLDL, oxLDL, PBS, P45 conjugated to BSA or BSA according to the manufacturer’s instructions.

**Plasma biochemistry**

Blood was collected in heparinized microtubes (Capiject; Terumo Medical, Elkton, MD, USA) and centrifuged at 4000 rpm for 10 min at 4°C. Plasma urea, creatinine, total calcium, phosphate and cholesterol were measured with a Modular P Hitachi automatic analyser (Roche A/S, Hvidovre, Denmark) and reagents from Roche A/S.

Plasma levels of interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) were measured with multiplex bead immunoassays (mouse cytokine 10-plex, LMC0001, Invitrogen, Taastrup, Denmark) and a Lumines platform.

**Antibody measurements**

Antibody (Ab) titres to Cu2+-LDL and MDA-LDL were measured by chemiluminescent ELISA as previously described [18]. Briefly, plasma samples obtained at indicated time points were diluted 1:100; and binding of immunoglobulin M (IgM) and IgG Abs to respective antigens was measured using alkaline phosphatase (AP)-conjugated goat-anti-mouse IgM and IgG Abs, respectively, followed by detection with LumiPhos. Total plasma IgM levels were measured in 1:10 000 diluted plasma sam-
Evaluation of atherosclerosis and plaque composition

The aortic arch from the heart to the sixth rib was opened longitudinally. Digital images of the luminal surface were acquired and analysed with the IM50 software (Leica, Copenhagen, Denmark). The en face plaque area was determined by dividing the atherosclerotic area with the total area of the aortic arch.

The formaldehyde-fixed heart with 1–2 mm of the aortic root was embedded in Tissue-Tek (Sakura Finetek Inc., Vaerloese, Denmark), and serial 10-µm cross-sections of the aortic sinuses were cut in a cryostate. With the appearance of the first aortic valve, the sections were collected on Super-Frost Plus slides (Menzel-Glaser, Germany). The sections were collected in series of three microscope slides (A–C) with three sections on each slide. Slide A contained sections number 1, 4 and 7; slide B sections number 2, 5 and 8; and slide C sections number 3, 6 and 9. In this manner, one A–C series spans an area of 90 µm, and each tissue section is separated by 30 µm on each slide. To quantify atherosclerosis, the sections were stained with oil-red-O (Sigma-Aldrich, O08625) and counterstained with Mayer's haematoxylin (Sigma-Aldrich, MHS-16). The cross-sectional plaque areas were quantified in a blinded manner by the same investigator in six sections from each mouse using IM50. To assess plaque morphology and cellular composition, the sections were stained for 1 h in 0.1% picrosirius red (Fluka, catalogue number 43665, dissolved in saturated aqueous picric acid). The sections were washed with acidified H2O2, dehydrated and mounted with Pertex. Smooth muscle cells and macrophages were detected with immunohistochemistry using α-actin (actin smooth Ab, clone 1A4; MS-113-B1; Neomarkers, Freemont, CA, USA) and Moma-2 (MCA519, AbD Serotec, Oxford, UK) antibodies. The α-actin was stained as previously described [19]. For detection of macrophages, the DAKO-strep-tABCComplex/HRP kit (Dakocytomation, Glostrup, Denmark) was used according to the manufacturer's instruction with the following modifications: Antigens were retrieved by a microwave oven treatment for 15 min in citrate buffer, pH 6.0 (10 mM citric acid), and endogenous peroxidase activity was blocked by incubation in 1% H2O2 for 10 min at room temperature (RT). After mounting in Shandon racks, the sections were blocked in 5% goat serum (in 0.25% BSA/TBS) for 30 min and subsequently incubated overnight at 4°C with Moma-2 antibody (1:100) diluted in 0.25% BSA/TBS. The sections were incubated for 2 h at RT with biotinylated rabbit anti-rat antibody (E468, Dakocytomation) diluted 1:200 in 0.25% BSA/TBS, developed with Nova Red and counterstained with Mayer's haematoxylin. Collagen in sections stained with picrosirius red was visualized under polarized light. The collagen, Moma-2 and α-actin staining areas were determined in two to three sections using the image analysis software, Visiomorph (Visiopharm, Hørsholm, Denmark).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA and Mann–Whitney tests were applied as stated in the manuscript. P < 0.05 was considered significant.

Results

Effect of immunization and 5/6 nephrectomy

ApoE−/− mice were immunized with PBS, nLDL, oxLDL, apoB-peptide P45 conjugated to BSA or BSA at the ages of 6, 9 and 11 weeks. OxLDL immunization caused a significant increase in plasma titres of IgG reacting with Cu2+-oxLDL and MDA-LDL but did not affect the corresponding IgM titres (Figure 1A, B and Supplementary Table 2). ApoB-peptide P45 immunization increased plasma titres of IgM reacting with Cu2+-oxLDL but did not affect plasma titres of IgG reacting with Cu2+-oxLDL and MDA-LDL or IgM reacting with MDA-LDL (data not shown). Plasma levels of the pro-atherogenic cytokine IL-12 [20,21] were lower in oxLDL-immunized mice than in nLDL-immunized mice (Table 1).

Subsequent to immunization, uremia was induced by 5/6 nephrectomy at the ages of 12 and 14 weeks. The increased plasma concentrations of urea, creatinine, calcium and phosphate indicated that 5/6 nephrectomy induced moderate uremia similar to what we have seen in previous studies [9,17] (Supplementary Table 3). Immunization with oxLDL and apoB-peptide P45 did not affect plasma markers of uremia, cholesterol or weight gain during the study (Supplementary Table 3).

Plasma anti-oxLDL antibody and cytokine concentrations in uremic mice

The plasma titres of IgG reacting with Cu2+-oxLDL and IgM reacting with Cu2+-oxLDL and MDA-LDL increased substantially after induction of uremia in all immunized groups (Figure 1A, B; Supplementary Table 2 and data not shown). Post-uraemia, the plasma titres of anti-MDA-LDL IgG were significantly higher in nLDL- and oxLDL-immunized mice than in mice that had received PBS (Figure 1B). ApoB-peptide P45 immunization did
not affect post-uraemia anti-Cu²⁺-LDL or anti-MDA-LDL plasma titres (data not shown). Also, the initial oxLDL immunization-specific increases in the plasma titres of IgG reacting with Cu²⁺-oxLDL and MDA-LDL were completely overruled by the massive effect of uraemia (Figure 1). Moreover, the post-uraemia IL-12 plasma concentrations were markedly increased above baseline levels and were similar in the PBS, nLDL and oxLDL groups (Table 1). Likewise, plasma levels of the potentially pro-atherogenic IL-2, IL-4 and IL-6 [20–23] increased upon induction of uraemia and were not affected by immunization with nLDL or oxLDL (Table 1).

Atherosclerosis

Atherosclerosis was quantified 16 weeks after induction of uraemia (at ∼30 weeks of age) both by en face analyses of the aortic arch and by histological examination of cross-sections of the aortic sinus. The extent of atherosclerotic lesions was almost identical in oxLDL-, nLDL- and PBS-immunized groups (Figure 2A, B). Also, immunization with the apoB-peptide P45 did not affect lesion size in uraemic mice (Figure 2A, B). Moreover, contents of smooth muscle cells, macrophages, smooth muscle cells and collagen within the plaques were similar in oxLDL-, nLDL- and PBS-immunized mice (Figure 3A, B).

Discussion

We examined whether the accelerated atherosclerosis caused by uraemia in apoE−/− mice could be attenuated by immunization with oxLDL prior to induction of uraemia. Immunization with oxLDL was successful as it increased plasma titres of IgG reacting with oxLDL and MDA-LDL were completely overruled by the massive effect of uraemia (Figure 1). Moreover, the post-uraemia IL-12 plasma concentrations were markedly increased above baseline levels and were similar in the PBS, nLDL and oxLDL groups (Table 1). Likewise, plasma levels of the potentially pro-atherogenic IL-2, IL-4 and IL-6 [20–23] increased upon induction of uraemia and were not affected by immunization with nLDL or oxLDL (Table 1).

### Table 1. Plasma cytokine levels post-immunization and post-uraemia (termination)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PBS</th>
<th>nLDL</th>
<th>oxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (pg/mL)</td>
<td>Post-immunization BD</td>
<td>154.7 ± 62.0</td>
<td>69.6 ± 37.7</td>
</tr>
<tr>
<td></td>
<td>Post-uraemia</td>
<td>69.6 ± 37.7</td>
<td>48.3 ± 16.9</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>Post-immunization BD</td>
<td>5.1 ± 2.6</td>
<td>69.6 ± 37.7</td>
</tr>
<tr>
<td></td>
<td>Post-uraemia</td>
<td>69.6 ± 37.7</td>
<td>48.3 ± 16.9</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>Post-immunization BD</td>
<td>11.26 ± 6.3</td>
<td>74.4 ± 57.0</td>
</tr>
<tr>
<td></td>
<td>Post-uraemia</td>
<td>74.4 ± 57.0</td>
<td>34.6 ± 16.8</td>
</tr>
<tr>
<td>IL-12 (pg/mL)</td>
<td>Post-immunization 145.7 ± 10.6</td>
<td>182.9 ± 13.6*</td>
<td>731.3 ± 125.9**</td>
</tr>
<tr>
<td></td>
<td>Post-uraemia</td>
<td>182.9 ± 13.6*</td>
<td>731.3 ± 125.9**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. BD, below detection limit of the assay (IL-2: 20 pg/mL; IL-4: 5 pg/mL; IL-6: 10 pg/mL). The plasma concentrations of IL-1β, IL-5, IL-10, GM-CSF, IFN-γ and TNF-α were below the detection limit of the assay both post-immunization and post-uraemia (data not shown). For IL-2, IL-4 and IL-6, there were no statistically significant differences between immunization groups. *P < 0.05, compared to oxLDL-immunized mice post-immunization (Mann-Whitney test). There were no statistically significant differences between IL-12 levels in the three groups post-uraemia. **P < 0.05, compared to post-immunization data for each immunization group (Mann-Whitney test).
mentary Table 1). Since we found no effect of immunization with either oxLDL or the apoB-peptide P45, the present data suggest that the marked pro-inflammatory and pro-oxidant effects of uraemia overrule the effect of immunization, at least in the 5/6 nephrectomized apoE−/− mouse model.

Even though immunization with oxLDL specifically increased plasma titres of IgG reacting with oxLDL, the magnitude of the increase was smaller than previously observed in MDA-LDL-immunized mice [18,24]. As such, it is possible that a protective effect of immunization in uraemic atherosclerosis may be achieved by using other immunization protocols (e.g. with MDA-LDL) which may produce more pronounced and sustained antibody responses. Nevertheless, immunization with both LDL and oxLDL did affect the immunological response to uraemia since the plasma titres of antibodies reacting with MDA-LDL increased more in the nLDL- and oxLDL-immunized groups than in the PBS group. Moreover, atheroprotective effects of immunization with nLDL, oxLDL and apoB-derived peptides have been achieved even without significant changes in titres of antibodies reacting with modified LDL [25,26]. As such, the present data support the conclusion that the protective effect of immunization with oxLDL in uraemic atherosclerosis indeed is absent or less pronounced than in classical atherosclerosis.

The marked increases in plasma titres of antibodies reacting with Cu2+-oxLDL and MDA-LDL upon 5/6 nephrectomy agree with previous studies in uraemic apoE−/− mice [10]. The production of oxLDL antibodies probably reflects that uraemia increases both oxidative stress [7] and formation of oxLDL [10]. Indeed, in LDL receptor-deficient mice, there is a positive correlation between plasma levels of oxLDL and antibodies reacting with oxLDL [27]. Cytokines mediate at least part of the downstream effect of oxLDL immunization. Thus, IL-5 has a pivotal anti-atherogenic role in the protective effect of MDA-LDL immunization of LDL receptor knockout mice [18]. During atherogenesis, specific cytokines have pro-inflammatory, pro-atherogenic roles (i.e. IL-2 and IL-12) [20,21,23,28], and others act in a strict anti-inflammatory and atheroprotective fashion (i.e. IL-5) [18,21], whereas yet others have variable roles depending on the surrounding environment (i.e. IL-4 and IL-6) [20–23]. Uraemic patients have altered levels of specific cytokines including increased IL-6 plasma concentrations [22]. The
Effect of uraemia overrules the potential of oxLDL immunization in apoE−/− mice

The present study revealed that uraemia in apoE−/− mice also leads to marked increases of plasma IL-2, IL-4, IL-6 and IL-12 suggesting that the uraemic apoE−/− mouse model may be suitable for further elucidation of the inflammatory response to uraemia. Nevertheless, since the potential beneficial effect of oxLDL immunization was overruled by the pro-inflammatory and pro-atherogenic effects of moderate uraemia in apoE−/− mice, the present study also emphasizes that further efforts are needed to identify effective anti-atherogenic preventions in uraemic patients. Such may include either means to preserve the effect of oxLDL immunization upon development of uraemia or dampening of the inflammatory response, e.g. by inhibiting signalling via specific receptors for angiotensin II [10] or advanced glycation end products (RAGE) [11].

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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