Natural antibodies of newborns recognize oxidative stress-related malondialdehyde acetaldehyde adducts on apoptotic cells and atherosclerotic plaques

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Abstract

Malondialdehyde acetaldehyde (MAA) adducts are generated under oxidative stress and shown to be highly immunogenic. Our aim was to investigate the recognition of MAA adducts by human natural antibodies in newborns before or at the time of full-term pregnancy. Plasma samples of pre-term (n = 11) and full-term (n = 36) newborns were enriched in specific IgM binding to MAA adducts compared with the maternal plasma IgM levels. Umbilical cord blood lymphocyte phage display library was generated to clone Fabs that specifically recognized MAA adducts without cross-reactivity to malondialdehyde. Fab clones from the antibody libraries of the pre-term and full-term newborns showed high sequence homology to the germline genes encoding the variable regions of antibodies, confirming that these Fabs represented the natural antibody repertoire of human fetuses. The MAA-specific umbilical cord blood Fabs bound to apoptotic human endothelial cells and the binding was efficiently competed with MAA adducts. The MAA-specific Fabs also recognized epitopes on advanced atherosclerotic lesions, and the uptake of infrared (IR)-labeled MAA-low-density lipoprotein by mouse J774A.1 macrophages was significantly reduced in the presence of these Fabs. In conclusion, MAA adducts were identified as one of the major antigenic targets for human natural antibodies already before the time of birth. MAA-specific natural antibodies are suggested to regulate apoptotic cell clearance starting from fetal development and to participate in the immunomodulation of atherosclerosis development during adulthood.

Keywords: innate immunity, oxidized low-density lipoprotein, phage display, reactive aldehydes

Introduction

Natural antibodies are primarily IgM isotype and are present in the circulation of all vertebrates. They bind with low affinity to a conserved repertoire of epitopes that have varying chemical composition (1). The production of natural antibodies is mainly confined to a certain B-lymphocyte subset, identified as B1a cells in mice (2, 3) and the corresponding human B-cell subset in umbilical cord blood and adult peripheral blood has a phenotype CD20+CD27+CD43−CD70− (4). A substantial portion of the natural antibody repertoire binds to epitopes generated as a result of lipid peroxidation. Malondialdehyde (MDA) and acetaldehyde are naturally occurring and biologically reactive aldehydes produced in the body under oxidative stress. Lipid peroxidation reactions of polyunsaturated fatty acids generate MDA, which can form adducts with proteins.
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by binding to ε-amino groups of lysines. In the presence of acetaldehyde, MDA reacts with it and produces a highly stable malondialdehyde acetaldehyde (MAA) adduct, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (MDHDC adduct shown in Fig. 1) (5–7). The formation of MAA adducts has been associated with alcohol consumption, alcoholic liver disease and smoking in humans and in animal studies (8, 9). MAA adducts are immunogenic even without adjuvants (10) and possess pro-inflammatory and profibrogenic properties (11), therefore they can be involved in the development and progression of atherosclerosis, as well as in the pathogenesis of alcoholic liver injury (8, 9, 12). Oxidation of low-density lipoprotein (LDL) is known to enhance the development of atherosclerosis. The lysine residues of apolipoprotein B on LDL particles can be modified by the reactive aldehyde adducts under conditions of lipid peroxidation. Both MDA- and MAA-modified LDL particles are found in the atherosclerotic lesions (13, 14) and are known to contain immunogenic epitopes recognized by the humoral immune system.

Several mouse studies have established that MDA adducts on LDL are recognized by natural IgM antibodies of innate immunity (15, 16). The natural IgM repertoire in human umbilical cord blood has also been shown to bind to MDA-LDL (17). However, there has been no report describing the human natural antibodies to MAA adducts. Furthermore, it has been reported that commercially available antibodies to MDA predominantly react with MAA adduct, and antibodies with similar reactivity to MAA are present in the sera of MDA immunized mice or high cholesterol fed rats (12). These data suggest that MAA is the dominant epitope after MDA modification of proteins or lipoproteins in atherosclerosis. Therefore, the question arises whether humans have natural antibodies against MAA epitopes already before birth e.g. to regulate tissue homeostasis by recognizing and removing particles with modified self-antigens such as apoptotic cell debris and oxidized LDL (OxLDL). The aim of this study was to investigate the recognition of MAA adducts by the natural antibody repertoire of newborns and to confirm the germline origin of the MAA-specific natural antibodies by cloning Fabs from both full-term and pre-term umbilical cord blood lymphocyte phage display libraries. Pre-term umbilical cord plasma and lymphocytes were included in the study in order to investigate the significance of innate immune recognition of MAA adducts already during fetal development.

Methods

Human samples

Umbilical cord blood samples were collected from pre-term (<32 weeks of gestation) and full-term (>36 weeks of gestation) neonates immediately after delivery. Maternal venous blood samples were collected 24–48 h after delivery in the Oulu University Hospital. Plasma was separated by centrifugation at 1700 × g for 10 min and umbilical cord blood lymphocytes were isolated with Ficoll-Paque Plus reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to manufacturer’s protocol. The study was approved by the ethical committee of the Oulu University Hospital, Finland (195/2006), and an informed written consent was obtained from each mother. Plasma total cholesterol, total triglycerides and high-density lipoprotein cholesterol were determined by enzymatic methods using commercial kits (Roche Diagnostics, Mannheim, Germany). LDL cholesterol was calculated using the formula of Friedewald et al. (18).

Isolation of LDL and modifications to LDL and bovine serum albumin

The LDL fraction (density 1.019–1.063 g ml⁻¹) was isolated from human plasma by sequential density-gradient centrifugation (19). MDA and MAA modifications to LDL and BSA

Fig. 1. Schematic formation of MAA-protein adducts. 2-Formyl-3-(alkylamino) butanal (FAAB adduct) is the rapidly degraded form of MAA adduct. MDHDC adduct, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde, is the stable fluorescent form of MAA adduct serving as the major biological target for recognition by antibodies (5–7). AA, acetaldehyde.
(Merck, Darmstadt, Germany) were prepared as described (20). Copper OxLDL (CuOx-LDL) was prepared by incubating LDL with 4 mM CuSO₄ for 24 h at 37°C. Reaction was stopped by adding EDTA to a final concentration of 200 µM, and LDL was dialyzed against 0.27 mM EDTA in PBS (21).

Chemiluminescence immunoassay with human plasma
Antigens (5 µg ml⁻¹) MAA-LDL, CuOx-LDL, phosphocholine-modified keyhole limpet hemocyanin (PC-KLH; Biosearch Technologies, Novato, CA, USA), total cell wall polysaccharide (CWPS; Statens Serum Institut, Copenhagen, Denmark), MAA-BSA, MDA-BSA and 0.5% (w/v) fish gelatin (Sigma, St Louis, MO, USA) were immobilized overnight at 4°C to 96-well microtiter plates in 0.27 mM EDTA in PBS. The wells were washed with an automated plate washer with 0.27 mM EDTA in PBS three times between each step of the immunoassays. Nonspecific binding sites were blocked with 0.5% fish gelatin and 0.27 mM EDTA in PBS for 50 min at room temperature and plasma samples (1:100) were incubated for 1 h at room temperature. In the competitive immunoassay, the plasma (1:100) was incubated with soluble MAA-LDL, CuOx-LDL, PC-KLH, CWPS, MAA-BSA, MDA-BSA and BSA (0–100 µg ml⁻¹) overnight at 4°C and centrifuged at 16 000 × g for 30 min at 4°C before adding the samples to microtiter plates with immobilized MAA-LDL, MAA-BSA or MDA-BSA. When measuring the total IgM concentration in plasma, 5 µg ml⁻¹ of anti-human IgM (Sigma) in 0.27 mM EDTA in PBS was immobilized to microtiter plates and purified human IgM (Sigma) was used as a standard. Plasma samples from the mothers were diluted 1:2000 and 1:10 000 and the plasma of the cord blood was diluted 1:400 and 1:800 for determining the total IgM concentration as an average obtained from the two different dilutions. Alkaline phosphatase-conjugated anti-human IgM (Sigma) was used as a secondary antibody and LumiPhos 530 as a substrate in the assay. The chemiluminescence was measured as relative light units (RLU) with a Wallac Victor™ multilabel reader (Perkin Elmer, Waltham, MA, USA).

Phage display library construction from umbilical cord blood lymphocytes
Total RNA from umbilical cord blood lymphocytes was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) and used to synthesize cDNA with M-MuLV reverse transcriptase and oligo(dT)₁₈ primers included in the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The pooled cDNAs from pre-term (n = 12) or full-term (n = 31) newborns were used for generation of antibody libraries. The two phage display libraries were constructed in three rounds of PCR with human Fab primers (22). Heavy chain variable regions, κ light chain variable regions and λ light chain variable regions were first amplified separately using cDNA as template. The constant regions of heavy chain, κ and λ light chains were amplified from the cloning human Fab template pComb3XTT and pComb3XO obtained from Dr CF Barbas at Scripps Research Institute, La Jolla, CA, USA. In the second-round PCR, the heavy and light chain overlap products were generated separately from the pooled first-round PCR products. The final full-length Fab coding fragments were assembled in the third PCR from the second-round products. The Fab coding fragments were digested with SfiI and cloned into the pComb3X phagemid vector. The precipitated and re-suspended ligation mixtures were transformed into XL1-Blue Escherichia coli (Agilent Technologies, Santa Clara, CA, USA) by electroporation (Gene Pulser® electroporator and cuvette with 0.2 cm gap; Bio-Rad, Hercules, CA, USA). After transformation, the bacteria were amplified and infected with VCSM13 helper phage (Agilent Technologies). Phage particles were obtained from the overnight culture medium by 4% (w/v) polyethylene glycol-8000/3% (w/v) sodium chloride precipitation and centrifugation at 15 000 × g for 15 min. Three successive rounds of panning against MAA-LDL were performed for umbilical cord blood lymphocyte phage display antibody libraries, and individual clones binding to MAA-LDL were selected with chemiluminescence immunoassay. The phagemid DNAs were isolated with QIAprep Spin Miniprep Kit (Qiagen). The nucleotide sequences were confirmed and aligned to the germline genes with the IMGT/V-QUEST sequence alignment tool (http://www.imgt.org) (23, 24).

Table 1. Genes encoding Vₜ and V₍ from Fab clones against MAA-LDL

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<th>Clones</th>
<th>Vₜ Identity (%)</th>
<th>V₄ Identity (%)</th>
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| 1      | IGHV4-b*01     | IGKV3-20*01    | 90.4
| 2      | IGHV4-b*01     | IGKV3-20*01    | 91.1
| 3      | IGHV4-b*01     | IGKV3-20*01    | 90.8
| 4*a    | IGHV4-b*01     | IGKV3-20*01    | 94.7
| 5      | IGHV4-b*01     | IGKV3-20*01    | 90.4
| 6      | IGHV4-b*01     | IGKV3-20*01    | 89.4
| 7      | IGHV4-39*01    | IGKV1-39*01    | 99.3
| 8      | IGHV4-39*01    | IGKV1-39*01    | 98.2
| 9      | IGHV4-39*01    | IGKV1-39*01    | 97.1
| 10     | IGHV4-39*01    | IGKV1-39*01    | 97.1
| 11*a   | IGHV4-b*01     | IGKV1-39*01    | 99.3
| 12     | IGHV4-b*01     | IGKV1-39*01    | 98.2
| 13     | IGHV4-39*01    | IGKV1-39*01    | 93.3
| 14     | IGHV4-39*01    | IGKV1-39*01    | 94.7
| 15     | IGHV4-4*02     | IGLV1-40*01    | 94.1
| 16     | IGHV4-39*01    | IGLV1-44*01    | 93.0
| 17     | IGHV3-23*04    | IGKV1-5*03     | 89.3

The clones selected for further characterization: Fab-pre was generated from clone 4 and Fab-ft from clone 11.
and phosphocholine-BSA (PC-BSA) (4 μg/ml; Life Technologies), 1× hypoxanthine/aminopterin/thymidine media supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA). 3–4 × 10⁵ cells were incubated with 2.5 μg ml⁻¹ of the affinity-purified Fab-pre or Fab-ft for 45 min at 4°C. Fab-specific anti-human-IgG-FITC conjugate (0.5 μg ml⁻¹; Sigma) was used as a secondary antibody. The binding of Fab antibodies to apoptotic EA.hy926 cells was determined with FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed with FlowJo 7.6.4 software (TreeStar, Ashland, OR, USA). The competitive binding of Fab antibodies to apoptotic cells was carried out by pre-incubation of Fab-pre or Fab-ft with polystyrene microparticles (6 μm; Polysciences Europe, Eppelheim, Germany) coupled with MAA-BSA or BSA overnight at 4°C.

Macrophage uptake assay
Mouse J774A.1 macrophages were maintained in DMEM containing 10% FBS, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin at 37°C with 5% CO₂. The uptake assay of MAA-LDL opsonized with Fab-pre and Fab-ft by J774A.1 macrophages was carried out on 96-well cell culture plates seeded with 10⁵ cells per well and cultured overnight. MAA-LDL was labeled with IRDye 800CW (LI-COR Biosciences) according to the provider’s instructions. IR800-conjugated MAA-LDL (0.5 μg ml⁻¹) in serum-free DMEM was incubated with macrophages in the presence or absence of the affinity-purified Fab-pre and Fab-ft (0, 12.5 and 25 μg ml⁻¹) for 3 h at 37°C. The wells were washed three times with PBS and analyzed with an Odyssey IR imaging system (LI-COR Biosciences). After analysis, the cells were lysed with 1 M sodium hydroxide overnight at room temperature, the amount of cell proteins was measured and the results were calculated as IR fluorescence normalized with the protein contents of each well.

Immunohistochemical staining of Fabs in the atherosclerotic lesions
Formalin-fixed paraffin-embedded aortic origin cross-sections from LDL receptor-deficient mice on a high fat diet were immunostained with umbilical cord blood Fabs specific for MAA epitopes. Antigen retrieval was performed by 10-min treatment with 10mM sodium citrate pH 6.0 near boiling point. The cross-sections were incubated with Fabs or PBS and stained with goat on rodent HRP Polymer Kit (Biocare Medical, Pike Lane Concord, CA, USA) according to the kit protocol using 3,3’-diaminobenzidine (DAB+) chromogen from Dako North America (Carpinteria, CA, USA). Briefly, endogenous peroxidase activity was quenched with Peroxidase Block (Dako North America). About 5 μg ml⁻¹ of Fab or PBS was applied onto separate sections followed by incubation with 2 μg ml⁻¹ secondary goat anti-human IgG Fab-specific antibody (Sigma). HRP-labeled polymer conjugated with anti-goat antibody and DAB+ chromogen were used for detection. Sections were further counterstained with Mayer’s hematoxylin. Images were acquired with Leica DM 3000 using Leica Application Suite (LAS) V4.1.0 (Leica Microsystems).

Statistical analysis
Statistical analyses were carried out using IBM SPSS statistics 19 software. The significances of the differences between the variables were compared with two-tailed t-test. \( P < \)
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0.05 were considered significant. The box-and-whisker plots represent quartiles, the median and the mean (black square). The whiskers denote the 10th and 90th percentiles and asterisks denote minimum and maximum values.

Results

Human umbilical cord blood contains natural IgM against the MAA adducts

Umbilical cord blood plasma and maternal plasma samples were analyzed for IgM binding to MAA-LDL, CuOx-LDL, PC-KLH, CWPS and gelatin with a chemiluminescence immunoassay. The total IgM concentration in samples was also measured, and the data were analyzed as the ratio of antigen-specific IgM to total IgM. Plasma IgM binding to antigens and total IgM concentrations of the study samples are shown in Supplementary Table 1, available at International Immunology Online. Both full-term (Fig. 2A) and pre-term (Fig. 2B) newborns had relative enrichment of plasma IgM binding to MAA-LDL compared with their mothers. IgM to CuOx-LDL was also slightly increased in newborns compared with their mothers, but the newborns did not have IgM to PC epitope or CWPS. Competitive immunoassays of umbilical cord plasma IgM binding were performed with MAA-LDL, CuOx-LDL, PC-KLH and CWPS (Fig. 2C and D). Specific binding of umbilical cord plasma IgM was observed to MAA-LDL in both full-term (Fig. 2C) and pre-term neonates (Fig. 2D). CuOx-LDL, PC-KLH or CWPS did not compete for plasma IgM binding to MAA-LDL. The dominant specificity of umbilical cord plasma IgM to MAA adducts was further investigated by comparing antibody binding to MAA-BSA and MDA-BSA. In addition, competition assays using MAA-BSA, MDA-BSA and BSA were performed. Specific binding of umbilical cord plasma IgM was observed to MAA-BSA in both full-term (Fig. 3A) and pre-term neonates (Fig. 3B). MDA-BSA competed very minimally with the umbilical cord plasma IgM binding to MAA-BSA most likely due to the presence of small

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Natural antibodies to MAA adducts adducts

Amount of MAA epitopes generated during the preparation of MDA-BSA (12). Umbilical cord plasma IgM binding to MAA-BSA was significantly higher than IgM binding to MDA-BSA as shown for full-term newborns in Fig. 3C. Also, pre-term umbilical cord plasma had significantly higher IgM binding to MAA-BSA than to MDA-BSA (data not shown). Soluble MAA-BSA or BSA did not compete for plasma IgM binding to MDA-BSA in full-term umbilical cord plasma (Fig. 3C). A dilution curve of full-term umbilical cord plasma IgM also demonstrated the dominant recognition of MAA adducts and only minimal binding to MDA-BSA and BSA (Fig. 3C). IgG binding to MAA-LDL and the total IgG concentrations were also measured from full-term newborns and their mothers. The ratio of antigen-specific IgG to total IgG concentration showed no relative enrichment of umbilical cord plasma IgG binding to MAA-LDL as compared with their mothers (data not shown). Plasma lipids and cholesterol levels of mothers and newborns were measured from all samples and are shown in Supplementary Table 2, available at International Immunology Online.

Umbilical cord blood Fab antibodies to MAA-LDL have high sequence homology with the germline genes

Umbilical cord blood phage display libraries of pre-term and full-term newborns were panned against immobilized MAA-LDL. The positive clones were selected, sequenced and analyzed by the IMGT/V-QUEST sequence alignment tool. The germline gene sequence analysis for 17 productive Fab clones is presented in Table 1 (clones 1–10 from pre-term and clones 11–17 from full-term libraries). The sequences of heavy chain variable region (VH) were derived mainly from 4-b*01 (47%) and 4–39*01 (41%) families (Fig. 4, left) with over 99% identities to the germline genes (Table 1). Five types of VDJ gene recombination were observed in VH from each library (data not shown). The 3–20*01 (47%) and 1–39*01/1D-39*01 (35%) were mostly utilized for light chain variable region (VL) sequences with some mutations (Fig. 4, right). The identities between the VL sequences and their germline counterparts were in a range of 89–99% (Table 1). The length and sequence in complementarity determinant...
regions 3 (CDR3s) of \( V_H \) from the pre-term library were much more conserved compared with that in CDR3s of \( V_H \) from full-term library (data not shown).

**Fab antibodies specifically recognize MAA epitopes**

Two Fab clones were selected for further characterization after sequence analysis: Fab-pre from pre-term and Fab-ft from full-term libraries (Table 1; clone 4: IFUp-08_107 and clone 11: IFUf-08_108). Both clones were transformed and expressed in bacterial host *E. coli* BL21(DE3). After purification with affinity columns, the Fab-pre and Fab-ft were analyzed by SDS–PAGE. One dominant band (over 90% pure) corresponding to 50 kDa under non-reducing conditions and two bands about 25 kDa under reducing conditions, representing heavy and light chains, respectively, were observed from both clones (Fig. 5A, left). The two Fabs were bound by Fab-specific anti-human-IgG antibody on western blot (Fig. 5A, right). The purified Fab-pre and Fab-ft recognized MAA-BSA but not MDA-BSA or PC-BSA on western blot (Fig. 5B), suggesting that they were highly specific to the MAA epitope without cross-reacting with the MDA epitope. Similar recognition of MAA-LDL, but not MDA-LDL or native LDL, was also observed when lipoproteins were analyzed on an agarose gel and stained with umbilical cord blood Fab (Fig. 5C). The binding specificities of Fab-pre and Fab-ft to oxidized lipid and protein epitopes were examined by direct binding (Fig. 6A and D) and competitive chemiluminescence.

**Fig. 4.** Antibody variable region gene segment usage of Fab clones derived from umbilical cord blood of newborns. Left panel: germline genes encoding the variable region of antibody heavy chains (\( V_H \)). Right panel: germline genes encoding the variable region of antibody light chains (\( V_L \)).

**Fig. 5.** SDS–PAGE and western blot analysis for purity and binding of the Fab-pre and Fab-ft. (A) One major band corresponding to 50 kDa (non-reduced) and two bands around 25kDa (reduced) were seen after purification from both clones (left panel, Pre: Fab-pre; Ft: Fab-ft). The same bands were recognized by Fab-specific anti-human-IgG antibody on western blot (right panel). (B) The purified Fab-pre and Fab-ft recognized MAA-BSA (lane 1 in two middle panels) but not MDA-BSA and PC-BSA (lanes 2 and 3 in two middle panels) on western blot. SDS–PAGE (left panel) and western blot with secondary antibody only (2ab) (right panel) are also shown. (C) Human lipoproteins were separated on agarose gel (upper panel) and transferred onto a nitrocellulose membrane before being stained with Fast Fat Red 7B. The membrane was cut into two from the middle of lane 3 and stained with Fab-pre and Fab-ft antibodies (bottom panel). Both Fabs recognized only MAA-LDL (lanes 1 and 5) but not MDA-LDL (lanes 2 and 4) and native LDL (lane 3).
Natural antibodies to MAA adducts added immunoassays (Fig. 6B, C, E and F). Both Fab antibodies showed binding only to immobilized MAA-LDL, not to MDA-LDL, CuOx-LDL, PC-KLH, CWPS or to native LDL (Fig. 6A and D). Competitive liquid-phase immunoassays demonstrated specificity to MAA-LDL and MAA-BSA for both Fabs (Fig. 6B, C, E and F). Partially competed binding of Fab antibodies to MAA-LDL was observed by pre-incubation with MDA-LDL (Fig. 6B and E) or MDA-BSA (Fig. 6C and F). It is most likely due to the existence of residual MAA epitopes in the MDA-modified preparations (12) rather than specific competition.

**Umbilical cord blood Fab antibodies bind to oxidized epitopes on apoptotic endothelial cells**

Oxidized lipid and protein epitopes are generated on cells undergoing apoptosis. The binding of umbilical cord blood Fab antibodies to modified self-antigens on apoptotic human vascular endothelial cells was tested. EA.hy926 cells were chosen for the study as they possess many of the characteristics of differentiated endothelial cell functions such as angiogenesis and inflammation (26). Fab-pre and Fab-ft showed similar binding to both early (Fig. 7A and C, gate R1) and late apoptotic cell populations (Fig. 7B and D, gate R2), whereas no binding was observed to non-apoptotic cells (Fig. 7B and E, gate R3). To examine the specificity of the binding to the MAA epitope, competitive immunoassays were performed by pre-incubation of Fab-pre or Fab-ft with polystyrene microspheres coupled with MAA-BSA or BSA before incubation with the apoptotic cells that were populated with DNA stain. The data indicated that the interactions between Fab antibodies and apoptotic endothelial cells were competed by MAA-BSA, but not by native BSA (Fig. 7F and G), suggesting that the Fab antibodies recognized certain epitopes with molecular mimicry to MAA epitope on apoptotic endothelial cells.

**MAA-specific Fabs reduce the uptake of MAA-LDL by macrophages and localize to atherosclerotic lesions**

The uptake of IR-labeled MAA-LDL by J774A.1 mouse macrophages was investigated in vitro in the presence and absence of umbilical cord blood Fabs. The results showed that both Fabs were able to partially block the uptake of MAA-LDL by macrophages in a dose-dependent manner (Fig. 8A). More efficient inhibition, up to 40% at a concentration of 25 μg ml⁻¹, was observed for Fab-pre (Fig. 8A, left). Fab-pre (Fig. 8C and E) and Fab-ft (Fig. 8D and F) demonstrated localization in the advanced atherosclerotic lesions most prominently in the intima and extending to the proximity of the media. These results further indicated that MAA-specific

![Fig. 6. Chemiluminescence immunoassays of human umbilical cord blood monoclonal Fab binding to oxidized epitopes. (A and D) Direct binding immunoassay for Fab-pre (A) and Fab-ft (D) to MAA-LDL, MDA-LDL, CuOx-LDL, PC-KLH, CWPS and native LDL. (B, C, E and F) Specific binding to MAA-LDL in competitive liquid-phase chemiluminescence immunoassay for Fab-pre (B and C) and Fab-ft (E and F). The binding is expressed as the binding in the presence of increasing amounts of soluble competitor divided by the binding in the absence of soluble competitor (B/B₀). The data are mean ± SD of triplicate determinations.](image-url)
Fig. 7. Flow cytometry analysis of the Fab-pre and Fab-ft binding to human endothelial cells. The early apoptotic cells were stained with annexin V and the cells in late stages of apoptosis were populated by DNA staining. R1 represents annexin V–binding early apoptotic cells (A), the late apoptotic cells are in R2 and the non-apoptotic cells in R3 (B). The background of cells only and the secondary antibody (2ab), and the binding of Fabs to early apoptotic cells (C), late-stage apoptotic (D) and non-apoptotic cells (E) were analyzed using anti-human IgG Fab-specific-FITC. The binding specificity to MAA epitope in DNA-stained R2 was demonstrated by pre-incubation of Fab-pre (F) or Fab-ft (G) with polystyrene microbeads coupled with MAA-BSA or BSA. **p < 0.01; ns, not significant. Two experiments were independently performed, and the results are the mean values ± SD of six replicates.
natural antibodies of newborns had an ability to recognize oxidative stress–related antigens and to regulate the uptake of OxLDL by macrophages in atherosclerotic plaques.

Discussion

MAA adduct is formed in the reaction of proteins with MDA and acetaldehyde. This adduct was first identified in the livers of rats after ethanol feeding (27), and later also found in atherosclerotic lesions of human aortas (14). MAA adducts bind auto-antibodies from serum of patients with alcoholic liver disease (28) and from animal models of atherosclerosis (12). MAA adduct has been proposed to be one of the main epitopes for the immune system after MDA modification of proteins in atherosclerosis (12). In this study, we established the biological presence of human natural antibodies binding to MAA adduct in umbilical cord plasma and showed that the IgM concentration recognizing MAA adducts in relation to total IgM was significantly greater in the newborns than in the mothers. Fab antibodies with germline identity were cloned with a phage display method from umbilical cord blood lymphocytes of neonates born even prior to 32 weeks of gestation or after full-term pregnancy. The Fab antibodies bound specifically to MAA adducts with no cross-reactivity to MDA adducts and revealed functional similarities to the previously described natural mouse IgM antibodies with ability to bind to apoptotic cells and inhibit the uptake of modified LDL by macrophages (29). This study discovered the existence of natural antibodies specific to MAA adducts even in pre-term neonates, and these natural antibodies demonstrated an ability to recognize oxidative stress–related, naturally occurring antigens on apoptotic cells and atherosclerotic plaques.

IgM antibodies binding to epitopes generated as a result of lipid peroxidation represent a substantial portion of the repertoire of natural antibodies. IgM antibodies of the cord blood, together with IgA, originate from the developing fetus itself as a germline-encoded repertoire of natural IgM. The placental transport of immunoglobulins allows only the transfer of IgG, but not IgM or IgA, through the placenta from mother to fetus (30, 31). The neonatal and fetal natural IgM showed dominant recognition of MAA adducts, minimal binding to CuOx-LDL and no binding to PC-KLH or CWPS, indicating that the MAA epitope would be one of the dominant targets for natural IgM that are present at the time of birth; this has also been detected in extremely pre-term deliveries (from gestational age 24+4). The relative enrichment of MAA-specific IgM in pre-term neonates was essentially similar to full-term neonates, which emphasizes the role of MAA-specific natural IgM already during fetal development. The exact reasons why MAA adducts were dominantly recognized by the natural repertoire of IgM are currently unknown. It could be partially due to the lack of exposure to microbial products and PC epitope in utero before birth and bacterial colonization. Further studies are needed to establish the comprehensive physiological explanation for the dominance of MAA adduct recognition, e.g., to explain why natural IgM in the fetus is not induced against MDA adducts, CuOx-LDL or epitopes cross-reactive with PC even though all these oxidatively modified epitopes are generated on apoptotic cells after birth in humans and animal models (32, 33).

From the umbilical cord blood lymphocyte Fab library of full-term and pre-term newborns, we cloned two Fab clones that were highly selective and specific for MAA-modified LDL or MAA-BSA, and neither the MDA modification nor the native LDL was recognized. This unique property allows them to be used for measuring specifically MAA adducts without cross-reactivity to MDA adducts. Both umbilical cord blood Fab clones showed high sequence homology (99.3–100%) with the germline genes encoding the V(H). It is noteworthy that cloning of the MAA-specific Fab also from the pre-term newborns provides further evidence supporting the similarity of the germline repertoire of natural IgM in full-term and pre-term newborns.
between full-term and pre-term newborns as observed on umbilical cord plasma IgM binding to MAA adducts.

The observations that Fab-pre recognizing MAA adducts was cloned from the lymphocytes of pre-term neonates and that the umbilical cord plasma from pre-term newborns contained MAA-specific IgM evoke the question: which antigens serve as targets for MAA-specific natural antibodies during fetal development before full-term pregnancy? The possible antigens originating from the fetus could be apoptotic cells because the process of apoptosis generates modified cell membrane lipid and protein structures that have been demonstrated to share molecular mimicry with e.g. MDA-adducted proteins (29). The polyclonal natural IgM repertoire is essential for the balanced clearance of apoptotic cell debris (34, 35) and certain IgM clones are devoted to regulate the specific immune responses in this process (36, 37). Moreover, the deficiency of natural IgM in a mouse model leads to impaired clearance of apoptotic cell (36, 38) and predisposes to a lupus-like syndrome with the development of auto-antibodies to nuclear antigens (39). Natural IgM plays an important role in controlling tissue homeostasis via participating in the clearance of apoptotic cells, inhibition of inflammation, removal of misfolded proteins and regulation of auto-antibody-producing B cells (40). In this study, we showed that the apoptosis of endothelial cells generated neoepitopes recognized by the MAA-specific umbilical cord blood Fabs, and the binding was efficiently competed by MAA-BSA. Thus, it can be hypothesized that structural remodeling during fetal development, in which apoptosis is known to be crucial for proper development, serves as one of the sources for antigens of MAA-specific natural antibodies. Especially, the remodeling of the fetal vasculature involves the apoptosis of endothelial cells, which further establishes the possible role for MAA-specific natural antibodies in the clearance of cellular debris in the fetus.

In addition to a particular physiological role in the fetus, the abundant IgM levels to MAA adducts may also be affected by various environmental factors. Epigenetic programming and maternal immunizations have been reported to alter fetal immune responses and IgM levels (41, 42). Also, recent observations indicate that maternal antigen exposure modulates even neonatal innate immune responses (43, 44) and specific adaptive responses (45, 46). The influence of maternal health status as a determinant of fetal IgM levels to MAA adducts should also be considered in future studies especially in terms of high fat diet, resulting maternal hypercholesterolemia and possible alcohol abuse during pregnancy. Previous studies have already shown that hypercholesterolemia of the mother increases the extent of fetal early fatty streak lesions compared with normocholesterolemic mothers (47) and influences the transplacental passage of normal and oxidized fatty acids (48).

Ox-LDL contains various different types of immunogenic epitopes derived from modified lipid and protein structures, but the exact epitopes for natural antibody binding remain relatively indefinite especially in the case of aldehyde adducts. MAA is a closely related structure to MDA and is suggested to be the dominant epitope after MDA modification in proteins or lipids. It is reasonable to speculate that the umbilical cord blood IgM antibodies to MDA-LDL described in one previous study (17) might actually represent the innate repertoire of natural IgM binding to both MDA- and MAA-LDL. LDL has been identified as one of the principal targets of umbilical cord IgM by antigen microarray studies (49). In addition, various other proteins and tissue components can be bound by umbilical cord blood IgM, e.g. blood clotting factors, heat shock proteins, immune modulators galectins and gelosolin, and single- and double-stranded DNA (49). Many of these targets of natural umbilical cord blood IgM are clinically important due to their associations with diseases like systemic lupus erythematosus, multiple sclerosis, type 1 diabetes, rheumatoid arthritis and atherosclerosis.

The umbilical cord blood Fabs to MAA adducts immunostained atherosclerotic lesions most prominently in the intima, and these Fabs were efficient in blocking the uptake of IR-labeled MAA-LDL by mouse macrophages in vitro. These findings emphasize the significance of the MAA adducts in the development of atherosclerosis in addition to the previously characterized PC and MDA epitopes on Ox-LDL and apoptotic cells. The MAA epitope has also been reported to be recognized by other components of the innate immune system such as the complement anaphylatoxin C3a (20) and the complement factor H (50). The activation of the complement cascade is enhanced in atherosclerotic lesions (51, 52). MAA-adducted proteins also induce T-cell responses in cell culture studies (53). An increased secretion of the pro-inflammatory cytokine TNF-α concomitant with the increased expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 have been reported after MAA adduct stimulation of rat heart endothelial cells (14). These pro-inflammatory and pro-adhesive changes by MAA adducts can also be attributed to various other cell types that participate in the development of advanced plaques in the arterial wall. Still, the exact role of natural antibodies to MAA adduct in the development of atherosclerotic lesions remains to be elucidated.

The chronic inflammatory processes throughout the lifespan may be regulated by joint action of several components of innate immunity according to the extent of oxidative stress and MAA epitopes generated in these processes. In this study, we established the role of MAA adducts as a significant target for the natural antibodies of humans. Natural antibodies binding to MAA adducts were already present during fetal development and they had functional properties related to the recognition and clearance of apoptotic cells and immunomodulation of atherosclerosis.

Supplementary data

Supplementary data are available at International Immunology Online.

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References


Natural antibodies to MAA adducts


