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Research paper

Development of anti-chloro ^{192} tyrosine HDL apoA-I antibodies for the immunodiagnosis of cardiovascular diseases

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ABSTRACT

High density lipoproteins (HDL) are considered cardio protective. Apolipoprotein A-I (apoA-I), a major component of HDL helps in reverse cholesterol transport, whose function is greatly affected during atherosclerosis due to oxidation by myeloperoxidase. Amino acid tyrosine residue of apoA-I at position 192 and 166 are sensitive to oxidation by myeloperoxidase resulting in the generation of chlorinated and nitrated apoA-I and they are believed to be present in atherosclerotic plaques and in circulation. These oxidized apoA-I have been suggested as potential indicator(s) of CVD risks in humans. To detect the levels of oxidized apoA-I there is a need for developing monoclonal antibodies (mAbs) with high specificity and sensitivity that could be utilized routinely in clinical immune based assays for blood plasma or for *in vivo* imaging. In this study, chemically chlorinated apoA-I (chlorinated ^{192}tyrosine- apoA-I) and a short synthetic peptide, containing the corresponding chlorinated tyrosine residue, conjugated to keyhole limpet hemocyanin (KLH) carrier protein were used for immunization. Stable hybridoma clones F7D5 and G11E3 were found to be highly sensitive and reactive towards chlorinated ^{192}tyrosine- apoA-I. Interestingly, these mAbs also displayed positive reaction with atherosclerotic plaques obtained from mouse and human biopsies. *In vitro* or *in vivo* diagnostic tests could be developed either by detecting oxidized apoA-I in human plasma or by directly imaging atheroma plaques as both mAbs were shown to stain human atheroma. The anti-chlorinated ^{192}tyrosine- apoA-I mAbs described in this study may have a high diagnostic potential in predicting CVD risks.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide. Atherosclerosis is one of the major causes of CVD initiated by lipid accumulation such as cholesterol and triglycerides, which develops into an atheroma plaque restricting blood flow of arteries and promoting thrombosis. This causes myocardial infarction and stroke leading to death (Libby et al., 2010; Falk, 2006). High Density Lipoprotein (HDL) is an important lipoprotein present in plasma involved in the removal of excess of cholesterol, essentially through its major apolipoprotein, the apolipoprotein A-I (apoA-I). High levels of HDL lower the CVD risks, thereby gaining a high cardio-protective value (Kontush, 2014).

In chronic inflammatory conditions like atherosclerosis, apoA-I undergoes oxidation and the amino acid residues get modified, which renders HDL dysfunctional along with apoA-I (Ueda et al., 2007; Zheng et al., 2004; Daugherty et al., 1994). Macrophages and neutrophils are important sources of oxidants that damage HDL. One pathway involves myeloperoxidase, a phagocyte heme protein that is expressed at high levels in human atherosclerotic tissue (Shao et al., 2005). Moreover, latest findings suggested that the quality of HDL is more important than quantity, as the oxidized modifications in HDL- apoA-I render the lipoprotein pro-inflammatory thereby promoting atherosclerosis (Bergt et al., 2004a, 2004b). Oxidized apoA-I serves as an indicator of dysfunctional HDL and these modifications are mainly seen at specific amino acid residues such as tryptophan, tyrosine, methionine and lysine. For instance tryptophan is hydroxylated, tyrosine can either get chlorinated or nitrated, methionine oxidizes to methionine sulfoxide

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and lysine gets carbamylated. Tyrosine chlorination and nitration may be physiologically significant because HDL or apoA-I exposed to hypochlorous acid or peroxynitrite generated through the myeloperoxidase system results in the oxidized modification of tyrosine 166 and 192 of apoA-I, which are most sensitive sites. Such chlorinated or nitrated apoA-I is dysfunctional and inefficient in removing cholesterol from cultured cells by the ATP binding cassette transporter A1 (ABCA1) pathway (Zheng et al., 2004; Daugherty et al., 1994). It is well documented that the atherosclerotic plaques contain these oxidized apoA-I at high level but not much information is available on the levels of oxidized apoA-I in plasma. Currently there is lack of efficient mAbs to detect the levels of oxidized apoA-I, which can be either utilized to functionalize contrast agents for *in vivo* imaging or routinely used in clinical settings in high throughput assays.

In order to achieve this, now we have generated newer mAbs against both 192-chloro-tyrosine containing synthetic peptide and full length apoA-I polypeptide. In this process, we identified two stable hybridoma clones F7D5 (generated against chloro-192-tyrosine apoA-I polypeptide) and G11E3 (generated against chloro-192-tyrosine synthetic pepide) secreting antibodies to be sensitive and highly specific to chlorinated ^{192}tyrosine apoA-I peptide/protein. Importantly, these stable hybridoma clones (F7D5 and G11E3) were characterized for their reactivity on slices of atheroma obtained from human coronary biopsies and from an animal model of atherosclerosis (ApoE-/- mouse under western diet) by Immunohistochemistry. Both clones recognized structures within human and mouse atheroma with high specificity, underlying the possibility of *in vivo* pre-clinical and clinical assays. We further discuss the utility of the mAbs in detecting chlorinated apoA-I in myocardial infarction patients, which may be a valuable marker of CVD risks in humans.

2. Materials and methods

2.1. Materials

pDNR LIB vector with *apoA-I* gene (Gene bank accession number – BC005380) was bought from Saflabs Pvt. Ltd. (Mumbai, India). pGEX4T-1 expression vector, competent *Escherichia coli*, BL21(DE3) strain was obtained from Novagen (Madison, WI); Ampicillin (HiMedia Lab. Mumbai, INDIA). Restriction digestion enzymes (*BamH*I and *Not*I), Vent *polymerase* and T4-DNA *ligase* were from NEB (New England Biolabs Boston, MA). Purified plasma apoA-I protein was a kind gift from Prof. G. M. Anantharamaiah, Division of Gerontology & Geriatric Medicine, University of Alabama at Birmingham, Birmingham. Trypsin from porcine pancreas was purchased from Sigma–Aldrich (St. Louis, MO, USA), Formic acid (FA) and Mass Spectrometry grade acetonitrile containing 0.1% FA were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Native apoA-I peptide, chlorinated and nitrated apoA-I peptides were custom made and procured from Life Tein, LLC (New Jersey USA). Freund's complete adjuvant, Freund's incomplete adjuvant, Dulbecco's Modified Eagle's medium (DMEM), hypoxanthine, azaserine, poly ethylene glycol (PEG), dimethyl sulfoxide (DMSO), fetal bovine serum, bovine serum albumin, anti-mouse IgG-conjugated with horseradish peroxidase were purchased from Sigma (St. Louis, MO). Non-fat milk was purchased from Hi Media (Mumbai, India) and Tetra methyl benzidine (TMB)/H_2O_2 was purchased from Genei (Bangalore, India). ELISA plates were purchased from Nunc (Roskilde, Denmark) and all other sterile plastic wares were purchased from Cellstar, Greiner Bio-one (Frickenhausen, Germany).

All animals used in the monoclonal antibody (mAbs) production were approved by the Institutional Animal Ethical Committee (IAEC-6th/22/dated 01.09.2012 CPCSEA, New Delhi). Concerning immunohistochemistry, animal experiments on ApoE-/- mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by the ethic committee of Bordeaux (CEEA50). Human tissue specimens were provided by Pr DUCASSE Eric, vascular surgeon at CHU Pellegrin. Human biopsies were harvested from patients having undergone an endarterectomy surgery after an acute vascular event. All of the clinical interventions took place at CHU Pellegrin Hospital (Bordeaux, France). All work with tissues from human subjects had been approved by the CPP committee (Comité de Protection des Personnes Sud-Ouest et Outre Mer) of Bordeaux and from the Research Ministry in France (Authorization number DC -2016- 2724). The CPP committee waived the need for patient written consent because surgical waste no longer attached to the person is considered “RES NULLIUS “. Patients were informed by the clinicians; if they did not express their opposition to research, the de-identified samples were immediately processed and embedded in paraffin (paraplast X-tra, Leica mycrosystems SAS,France).

The different buffers used for Immunochemistry assays are composed of PBS, BSA protein (Eurobio life sciences, France), Triton X100 and Tween 20 (Sigma aldrich (France) nonionic surfactants.

Trapping of endogenous mouse immunoglobulins of mouse specimens was performed with anti-mouse IgG (H + L) antibodies (Beckman Coulter, France). The binding of anti-chloro ^{192}tyrosine HDL- apoA-I antibodies on mouse and human specimen sections was detected by secondary HRP - conjugated goat anti-mouse (Fcγ specific) antibody (Jackson Immunoresearch, PA, USA) and immunostaining-DAKO rabbit Envision plus kit (DAKO France SAS, France), respectively. The staining was performed by adding the peroxidase substrate diaminobenzidine (DAB substrate kit, DAKO France SAS, France). The slices were counterstained with hematoxylin (DAKO France SAS, France). The slices were dehydrated and mounted with mounting medium Coverquick 2000 (VWR prolabo Chemicals, France).

2.2. Construction of recombinant apoA-I

The pDNR-LIB vector containing the *apoA-I* gene was used as a template for the amplification of target DNA fragment through Polymerase Chain Reaction (PCR), using forward (5′ATGGATCCGATG AACCCCCCCAGAGC3′) and reverse (5′ATGCGGCCGCTCACTGGGTGT TGAG3′) primers with specific restriction sites (*BamH1 and Not1*). The PCR conditions were 92 °C - 2 min, denaturation 92 °C – 30 s hold, 68.7 °C – 30 s annealing, 72 °C –1 min hold, 72 °C – 20 min final extension, end at 4 °C. The reaction was carried out for 35 cycles with 0.2 mM dNTPs, 5 units of Vent *Polymerase*, 20 ng of template DNA (pDNR-LIB vector with *apoA-I* gene). The *BamH*I and *Not*I restriction sites were incorporated in the forward and reverse primers of *apoA-I* gene. The purified *apoA-I* DNA after restriction digestion was cloned into bacterial expression vector pGEX4T-1 having N-terminal GST polypeptide (GST- apoA-I fusion protein). The putative bacterial clones were verified first by restriction enzyme digestion followed by nucleotide sequencing (Sambrook and Russell, 2001).

2.3. Protein expression and purification of recombinant GST- apoA-I

E. coli BL21DE3 strain was grown in Luria and Bertani (LB) medium containing sodium chloride (10 g/l), peptone (10 g/l) and yeast extract (5 g/l). Expression of recombinant apoA-I was optimized at various temperatures with increasing concentrations of IsoPropyl β-D-ThioGalactoside (IPTG). Cell pellets were resuspended in lysis buffer (TrisHCl-50 mM, pH 8; NaCl 150 mM, Glycerol 5%, PMSF 1 mM, DNase 10 mg/ml, Lysozyme 10 mg/ml) for 1 h at 4 °C with gentle shaking for periplasmic extraction of recombinant protein. The cell lysate was centrifuged at 6300*g* at 4 °C for 25 min. The expression of the protein was analyzed on 12%-SDS-PAGE and confirmed by Western blot (Laemmli, 1970).

The expressed recombinant GST- apoA-I was purified by GST affinity chromatography. The soluble protein was loaded onto 1 ml GST affinity column (Glutathione Sepharose™ 4B, GE Healthcare) connected to a fully automated AKTA FPLC system (Amersham Bioscience, Uppsala, Sweden) consisting of a P-920 pump and a UPC - 900 monitor for measurement of UV absorption (wavelength set at 280 nm), pH and conductivity. The column was equilibrated with the binding buffer PBS pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) at a flow rate of 1 ml/min before loading the sample. After injecting the sample, all the non-retained proteins were washed with the binding buffer till the baseline was achieved. The target protein was eluted with 50 mM Tris (pH 9.0) containing 10 mM reduced glutathione. The protein concentration was determined by Bradford method (Bradford, 1976) and the protein fractions were analyzed on 12% SDS-PAGE followed by western blot. The sequence coverage and molecular mass of the expressed recombinant apoA-I were analyzed by Agilent Technologies 6540 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS.

2.4. Generation of polyclonal antibody to recombinant GST- apoA-I

The laboratory-bred female New Zealand albino rabbits were immunized with purified recombinant GST- apoA-I. The purified antigen (100 μg) was emulsified with Freund's complete adjuvant and the rabbits were immunized intra-dermally. Two booster doses of the antigen (100 μg) with Freund's incomplete adjuvant were given at an interval of 4 weeks. Serum samples were collected 10 days after the final booster and tested for immunoreactivity against the recombinant GST- apoA-I antigen by indirect ELISA. The antiserum containing the polyclonal antibody was purified and used for detecting the apoA-I.

2.5. Separation of GST tag from the recombinant GST- apoA-I protein

The purified fused GST tagged recombinant apoA-I (approx.1 mg) was subjected to digestion by thrombin using CleanCleave Thrombin Kit (Sigma–Aldrich, St. Louis, MO, USA) to remove the GST tag from the fusion protein, as a thrombin cleavage site was engineered between the GST tag and the protein of interest. The recombinant apoA-I was treated with thrombin-agarose suspension for 8 h at 28 °C and further, the mixture of supernatant from thrombin clean cleavage kit was passed again into a GST affinity column (GE healthcare) connected to a fully automated AKTA FPLC system. Here, apoA-I was obtained in the non-retained fraction (*i.e.* Binding buffer PBS 7.4) and the bound GST tag were eluted in elution buffer (50 mM Tris-HCl pH 9.0) containing 10 mM reduced glutathione.

2.6. Chemical modification of ^{192} tyrosine residue in apoA-I

The recombinant apoA-I (5 μg) was treated with 0.1 mM sodium hypochlorite in PBS pH 7.4 (140 mM NaCl, 2.7 mM, KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4) for 20 min at 4 °C and the reaction was terminated with the addition of 20 mM methionine (Bergt et al., 2004a, 2004b). After termination of the reaction, the modification of tyrosine into chlorotyrosine at position 192 in the apoA-I was analyzed and confirmed by mass spectrometric analysis (see below).

2.7. Mass spectrometric analysis of apoA-I for the modification of ^{192} tyrosine by LC–MS/MS (ESI-Q-TOF)

Native apoA-I and apoA-I treated with sodium hypochlorite were incubated for 8 h at 37 °C with sequencing grade modified trypsin at a ratio of 25:1 (*w*/w) protein/trypsin in 100 mM NH_4HCO_3, pH 8. Digestion was halted by acidification (pH 2–3) with trifluoroacetic acid. The samples were analyzed by LC–MS/MS on 1290 Infinity LC system consisting of a binary pump, temperature controlled auto sampler, thermo stated column chamber, diode array detector coupled with an 6540 UHD accurate-mass Q-ToF mass spectrometer equipped with Agilent Jet Stream Technology ESI source (Agilent Technologies, Santa Clara, CA, USA). Trypsinized native apoA-I and chemically chlorinated ^{192} tyr- apoA-I were injected and subsequently eluted from a reverse-phase column (Agilent Zorbax RRHD SB C-18 column, 2.1 mm × 150 mm, 1.8 μm particle size) at a flow rate of 0.2 ml/min. The mobile phase solvent A (0.1% HCOOH in H_2O) and solvent B (0.1% HCOOH in 100% CH3CN) were used. Solvent B was kept at a linear gradient from 0 to 100% for 45 min. The following conditions were used in the Agilent QTOF mass spectrometer. Gas temperature: 200 °C; drying gas: 5 L/min; nebulizer: 40 psig; sheath gas temperature: 350 °C; sheath gas flow: 11 L/min; VCap voltage: 3500 V; nozzle voltage: 1000 V; fragmentor voltage: 175 V. The LC–MS/MS system was operated using Agilent Mass Hunter B.04.00 software (Agilent Technologies, Santa Clara, CA, USA) in which the data acquisition was done by Mass Hunter Workstation B.04.00 and Mass Hunter Qualitative Analysis B.04.00 was used for the data analysis (Prasanna et al., 2014).

2.8. Peptide design and conjugation to carrier protein

Synthetic peptide of 7 amino acid length (189-LAE- $\mathbf{Y}_{\mathbf{Cl}}$- HAK-195) containing chlorinated tyrosine residue at position 192 of apoA-I conjugated with 2 different carrier proteins, Keyhole Limpet Hemocyanin (KLH) and Bovine serum albumin (BSA) through their cysteine in N-terminal, were custom ordered and purchased from LifeTein, LLC (New Jersey USA). Similarly native peptide without chlorinated ^{192} tyrosine residue and Ovalbumin (OVA) conjugated chloro tyrosine and nitro tyrosine residue at position 166 of apoA-I was also procured from LifeTein, LLC (New Jersey USA). The KLH conjugated chlorinated apoA-I peptide was used for mice immunization. While, the BSA conjugated chlorinated and non chlorinated apoA-I peptide and OVA conjugated with chlorinated and nitro tyrosine apoA-I peptide were used for the screening of hybridoma clones to identify positive and stable clones secreting mAbs reactive to chlorinated apoA-I.

2.9. Development of murine monoclonal antibodies

Monoclonal antibodies were developed by following the methods as described in our earlier work (Lokeshwaran and Krishnan, 2016; Rajak et al., 2013). Briefly, female BALB/c mice were immunized subcutaneously with 100 μg of chlorinated ^{192} tyrosine apoA-I protein and KLH conjugated chlorinated ^{192} tyrosine apoA-I peptide separately emulsified in Freund's complete adjuvant at the ratio 1:1. Booster injections were given twice at an interval of three weeks, with 100 μg of the same antigens emulsified in Freund's incomplete adjuvant. Three days prior to fusion, the mice were injected intraperitoneally with 200 μg of antigens in saline. Splenocytes were fused with Sp2/0 myeloma cells using 50% polyethylene glycol and 10% DMSO. The screening of hybridomas secreting mAbs were done by indirect ELISA using microplates coated with native apoA-I protein, chemically chlorinated ^{192} tyrosine apoA-I protein, BSA conjugated chlorinated ^{192} tyrosine apoA-I peptide and non-chlorinated (native) apoA-I peptide used as a control. The positive clones were expanded and subsequently sub cloned to monoclonality by the method of limited dilutions.

2.10. Enzyme Linked Immunosorbent Assay (ELISA)

The hybridoma clones were screened by indirect ELISA using microtiter plates coated with 1 μg/well of antigen (native apoA-I protein, chemically chlorinated ^{192} tyrosine apoA-I protein and BSA conjugated chlorinated ^{192} tyrosine apoA-I peptide, and BSA conjugated non chlorinated ^{192} tyrosine apoA-I peptide as a control) suspended in 100 mM carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. The plates were then washed with PBST (50 mM PBS with 0.1% tween-20) and blocked with 5% non-fat skim milk for 2 h at 37 °C. After three washes, cell culture supernatant was added and incubated for 1 h at 37 °C. The bound antibody was detected using goat anti-mouse IgG-HRP conjugate with tetra methyl benzidine (TMB)/H_2O_2 as the enzyme substrate. The reaction was stopped with 2 M sulfuric acid and absorbance was measured at 450 nm using multiwell plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany).

2.11. Characterization of the two mAbs generated against chlorinated ^{192} tyrosine apoA-I protein or synthetic chlorinated ^{192} tyrosine apoA-I peptide

2.11.1. Dot blot and Isotyping

The specificity of the murine mAbs generated to chlorinated ^{192} tyrosine apoA-I protein and synthetic chlorinated ^{192} tyrosine apoA-I peptide was confirmed by dot blot analysis on purified mAbs. Briefly, 3 μg of proteins (native apoA-I protein, chemically chlorinated ^{192} tyrosine apoA-I protein and BSA conjugated chlorinated ^{192} tyrosine apoA-I peptide, and BSA conjugated non chlorinated ^{192} tyrosine apoA-I peptide as a control) were spotted on the nitrocellulose membrane. The spotted samples were allowed to dry and then blocked in 5% skimmed milk solution. After incubation, the membrane was washed thrice with PBST and incubated for 1 h with selected mAbs in the dilution of 1:2000. After extensive washing with PBST, horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin was added and incubated for 1 h. The positive reactivity was visualized by using 3, 3′-Diaminobenzidine (DAB) as a chromogenic substrate and the images were captured using BIORAD ChemiDoc Imaging System.

The isotyping of the mAbs were determined using a mouse monoclonal antibody isotyping Kit (Pierce, IL, USA) according to manufacturer's instructions.

2.11.2. Purification of the two mAbs generated against chlorinated ^{192} tyrosine apoA-I protein and synthetically derived chlorinated ^{192} tyrosine apoA-I peptide

The cell culture supernatant containing secreted antibody was directly introduced into the MEP (4-Mercapto-Ethyl-Pyridine) hypercel column (Pall life science). For F7D5 mAb, the column was equilibrated with 50 mM phosphate buffer pH 7.4 and the bound proteins were eluted by decreasing the pH in step gradient with 50 mM sodium acetate buffer of pH 5.0, 4.0 and 3.0 respectively. For G11E3 mAb the column was equilibrated and washed with 0.1 mM sodium acetate, 500 mM NaCl pH 5.5. Then the remaining unwanted proteins came out in 0.05 mM sodium acetate (pH 4.8) and the pure antibody was eluted in 100 mM sodium acetate pH 3. The purity of the purified mAb was analyzed by 10% SDS-PAGE stained with Coomassie brilliant blue (Guerrier et al., 2000).

2.11.3. Antigen sensitivity of mAbs generated to chlorinated ^{192} tyrosine apoA-I peptide and protein

The monoclonal antibodies were characterized based on antigen sensitivity by coating different quantities (from 1000 ng to 0.9 ng per well) of the antigens (chlorinated ^{192} tyrosine apoA-I and native apoA-I proteins, BSA conjugated chlorinated ^{192} tyrosine apoA-I and BSA conjugated non chlorinated ^{192} tyrosine apoA-I peptides) on ELISA plate. The purified mAb (1 μg/0.5 mL) was used as a primary antibody followed by the secondary antibody; finally it's detected through by enzyme substrate. The reaction was stopped with 2 M sulfuric acid and absorbance was measured at 450 nm using multiwell plate reader.

2.11.4. Cross reactivity of mAbs with different modified apoA-I peptides

The selected monoclonal antibodies were tested for cross reactivity with four different modified apoA-I peptides containing chlorination and nitration of tyrosine residues at sites 192 and 166 respectively. The four different modified apoA-I peptides (KLH conjugated chloro-tyrosine 192(N terminal -189-C-LAE-3-**YCl**-HAK -195- C terminal), KLH conjugated nitro-tyrosine 192(N terminal -189-C-LAE-3-NO_2-**Y**-HAK -195- C terminal), OVA conjugated chloro-tyrosine 166 (N terminal -153-C-RAHVDALRTHLAP-3-**YCl**-SDELRQ-172-C terminal) and OVA conjugated nitro-tyrosine 166 (N terminal -153-C-RAHVDALRTHLAP-3-NO_2-**Y**-SDELRQ-172-C terminal) were serially diluted starting from 1000 ng to 0.9 ng per well and probed with respective purified primary antibodies at a dilution of 2 μg /ml by indirect ELISA method. Followed by the bound antibody were detected using goat anti-mouse IgG-HRP conjugate with tetra methyl benzidine (TMB)/H_2O_2 as the enzyme substrate. The reaction was stopped with 2 M sulfuric acid and absorbance was measured at 450 nm using multiwell plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany).

2.11.5. Immunohistochemistry on mouse aorta and human endarterectomy sections

Immunoreactivity of anti-chloro ^{192}tyrosine- apoA-I antibodies were evaluated on paraffin-embedded atheromatous mouse and human sections prepared from atheromatous specimens. Each paraffin embedded specimen was deparaffinised and rehydrated. Blocking steps (H_2O_2 blocking and unspecific site blocking with PBS/1% BSA/0.2% Triton X100) and a retrieval step (Tris-Hcl 10 mM, EDTA 1 mM, 0.05% Tween 20, pH 9.0) were performed. A supplementary blocking of mouse sections was performed to trap endogenous immunoglobulins with an anti-mouse IgG (H + L) at 100 μg/ml. After washing in PBST (PBS 1X, 0.025% TritonX100), slices were then incubated with purified anti-chloro ^{192}tyrosine- apoA-I antibodies (G11E3 and F7D5 mAbs) at 10 μg/ml overnight at 4 °C. Specimens were washed with PBST. Mouse specimens were then incubated with the secondary HRP-conjugated goat anti-mouse (Fcγ specific) antibody (1:1000). For human sections, the immunostaining-DAKO rabbit Envision plus kit was used. Negative control slices were incubated with the secondary antibodies only. After a further three washes with PBST, staining was performed by adding the peroxidase substrate diaminobenzidine with H_2O_2. After a wash in dH_2O to stop the enzymatic reaction, slices were counterstained in hematoxylin, dehydrated and mounted.

3. Results

3.1. Construction of expression vector for recombinant apoA-I

The pDNR-LIB vector containing the a*poA-I* cDNA clone was used as a template for the amplification of target DNA fragment through Polymerase Chain Reaction (PCR). The amplicon obtained was digested with specific restriction endonucleases *BamH*I and *NotI*. The apoA-I expression cDNA construct was generated by ligating the 750 bp amplicon into the expression vector pGEX4T-1 (containing N-terminal GST tag). Plasmids isolated from the positive transformants were digested with *BamH*I and *NotI* to confirm the presence of 750 bp insert of *apoA-I* gene. Then the positive clones were further confirmed through DNA sequencing.

3.2. Expression and purification of recombinant apoA-I

The recombinant expression vector was transformed into *E.coli BL21DE3* strain to express the recombinant apoA-I fused with GST tag. The IPTG concentration of 20 mM at 37 °C gave the best expression level. The expressed protein was confirmed by analyzing the molecular weight on a 12% SDS-PAGE gel. The molecular weight of apoA-I is 28.3 kDa but the expressed recombinant protein had higher molecular weight due to its fusion with GST tag of 26 kDa. The resulting migration of the fusion protein in the SDS-PAGE analysis was ~54 kDa. This expressed recombinant fusion GST- apoA-I was purified using the Glutathione Sepharose™ 4B (GE Healthcare) 1 mL GST affinity column (Fig. 1). Binding of *E.coli* protein lysate expressing recombinant GST-apoA-I was done using PBS at pH 7.4 and for the elution, 50 mM Tris-HCl pH 9.0 containing 10 mM reduced glutathione was used. (Fig. 1A) represents the chromatogram in which peak 1 indicates flow through or the unbound fraction and peak 2 is the protein eluted fraction. The corresponding fractions were further analyzed by 12% SDS-PAGE and a band corresponding to the eluted fraction is clearly visible at ~ 55 kDa (Fig. 1B). We further validated the purified fraction using in-house rabbit polyclonal anti- apoA-I antibody which recognized the apoA-I protein (Fig. 1C).

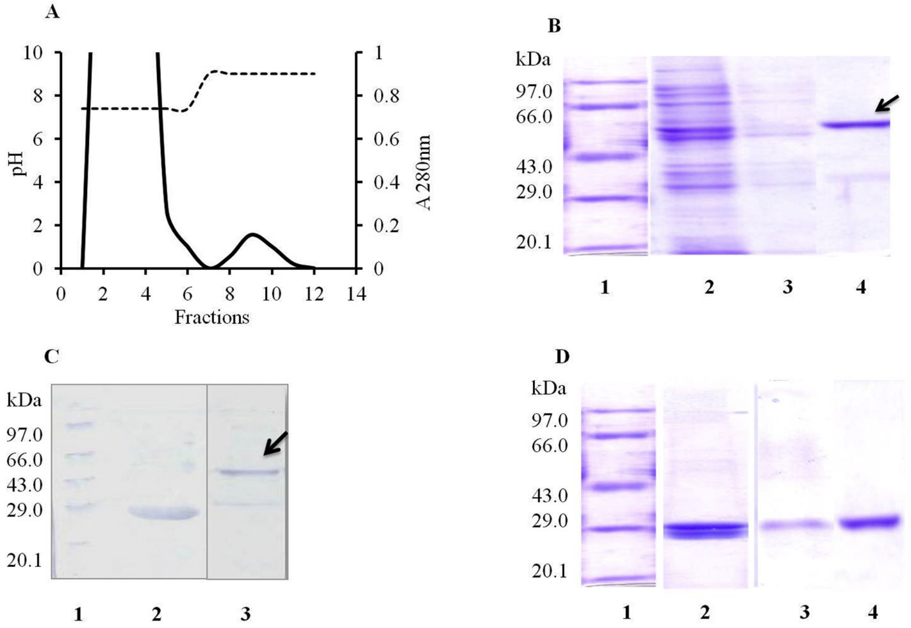

Fig. 1. Purification and validation of recombinant apoA-I. (A) Chromatogram depicting the purification of recombinant GST- apoA-I by GST affinity column. The soluble fraction (8 mg) ofbacterial cell lysed soup containing the GST tagged recombinant expressed protein was loaded into the column which was equilibrated with the binding buffer PBS, pH 7.4 at a flow rate of 1 ml/min and subsequently washed with binding buffer to remove non retained fractions in the column. The bound proteins were eluted with 50 mM Tris (pH 9.0) containing 10 mM reduced glutathione and the purified apoA-I amount was found to be 0.65 mg. (B) SDS-PAGE analysis of the purified of recombinant GST- apoA-I. Lane 1- Protein molecular weight marker, Lane 2 –Injected load, Lane 3- unbound protein in washed fraction, Lane 4 – elution fraction. The arrow indicates purified recombinant GST- apoA-I protein at pH 9.0. Samples were run on 12% acrylamide gel under non reducing condition except for Lane 1. (C) Confirmation of recombinant GST- apoA-I by Western blot. Lane 1- Protein molecular weight marker, Lane 2- Native apoA-I, Lane 3- Purified recombinant GST- apoA-I. Samples were run on 12% polyacrylamide gel under non reducing condition and transferred on to nitrocellulose membrane. Immunoblotting was performed with in-house anti- apoA-I pAb (1:8000) used as primary antibody and anti-IgG conjugated with horse radish peroxidase was used as a secondary antibody. The arrow indicates purified recombinant GST- apoA-I protein. (D) Analysis of cleavage of GST tag from recombinant apoA-I. The purified GST fused apoA-I protein was treated with a suspension of thrombin coupled-agarose which is designed to cleave the GST tag from the fusion proteins (see Section 2.5). Lane 1 – Protein molecular weight marker, Lane 2 – Cleavage of apoA-I from GST tag using CleanCleave Thrombin Kit from sigma (approximately 28 kDa and 26 kDa respectively), Lane 3 – apoA-I eluted in non retained fraction, Lane 4 – GST tag alone eluted in 50 mM Tris pH 9.0 containing 10 mM reduced glutathione. All samples were analyzed with 12% SDS-PAGE gel under non reducing condition except for Lane 1.

3.3. *Removal of GST tag from the recombinant GST- apoA-I protein by using thrombin followed by purification of the recombinant apoA-I protein without the tag*

After the purification of GST tagged recombinant apoA-I, 1 mg of purified recombinant protein was treated with thrombin to remove the GST tag. The Thrombin CleanCleave Kit ™ (Sigma) contains a 50% suspension of thrombin-agarose beads designed for cleavage of recombinant fusion proteins. The thrombin treated supernatant was loaded into GST affinity column (GE healthcare) connected to a fully automated AKTA FPLC system. Here, apoA-I (~28 kDa) was obtained in the non retained fraction (*i.e.* binding buffer PBS 7.4) and the bound GST tag was eluted in elution buffer 50 mM Tris-HCl (pH 9.0) containing 10 mM reduced glutathione. Then the fractions were analyzed on a 12% SDS-PAGE for confirmation of GST tag removal from apoA-I (Fig. 1D). The purified recombinant apoA-I protein was then subjected to chlorination by using the chemical sodium hypochlorite (see below).

3.4. *Validation of chlorinated ^{192} tyrosine apoA-I full length protein by LC–MS/MS (ESI-Q-TOF)*

The recombinant apoA-I protein was treated with 0.1 mM sodium hypochlorite. Then the chlorinated apoA-I was trypsinized and mass spectrometric analysis carried out as described in methods. Subsequently ms/ms analysis was done using Agilent 6540 LC-ESI-MS Q-TOF to confirm the presence of 3-chlorotyrosine at position 192 in the apoA-I amino acid sequence. The tryptic peptides subjected to MS/MS analysis revealed the presence of a peptide which represented the ^{192} tyrosine residue in apoA-I (N terminal – ^{189}-LAEYHAK -^{195}- C terminal). Compared to normal apoA-I this particular peptide sequence showed modification of ^{192} tyrosine into chlorotyrosine and a difference of 35 Da was observed in the molecular mass of the peptide. In (Fig. 2A and B), the peaks determined the molecular mass variation between the native and chlorinated apoA-I. (Table 1) shows the changes in the molecular mass of the peptide observed between unmodified and modified apoA-I by representing the y-ions obtained when the peptide is subjected to fragmentation starting from C-terminal and moving towards N-terminal in the MS/MS analysis. The total molecular mass of the peptide is 830 Da $[M + H]^+$ after obtaining single charge (H^+) in unmodified condition which increases to 865 Da in the modified peptide due to addition of chlorine atom in tyrosine residue at 192 position. This addition of chlorine atom in the tyrosine residue is represented by y4 ion where the molecular mass increases from 518 Da to 552 Da, an increase by 35 Da representing the peptide sequence N – YHAK – C. This molecular mass shift begins from y4 ion N – YHAK – C and continues all the way till y7 ion representing the entire peptide sequence N-LAEYHAK- C starting from C-terminal and ending towards N- terminal. This shift in the molecular mass of the peptide confirms the modification of tyrosine residue at position 192 into chlorotyrosine in apoA-I after *in vitro* modification using sodium hypochlorite.

3.5. *Synthetic chlorinated ^{192} tyrosine apoA-I Peptide*

Two synthetic peptides each 7 amino acids in length representing the tyrosine residue at position 192 in apoA-I (N term -189-LAEYHAK -195- C term) were custom synthesized and in one of the peptide the 192 tyrosine residue was chlorinated (N term -189-LAE-YCl -HAK -195- C term). The purpose of this synthetic peptide was to develop a monoclonal antibody with high specificity and sensitivity towards the chlorinated tyrosine residue and compare its specificity and sensitivity with the mAbs generated against chlorinated apoA-I protein (chemically chlorinated 192 tyrosine apoA-I protein).

3.6. *Production of monoclonal antibodies against chemically chlorinated ^{192} tyrosine apoA-I protein and synthetic chlorinated ^{192} tyrosine apoA-I peptide*

Both antigens (chemically chlorinated ^{192} tyrosine apoA-I protein and synthetic chlorinated ^{192} tyrosine apoA-I peptide) were used for immunization as described in methods (Section 2.9). Native apoA-I and

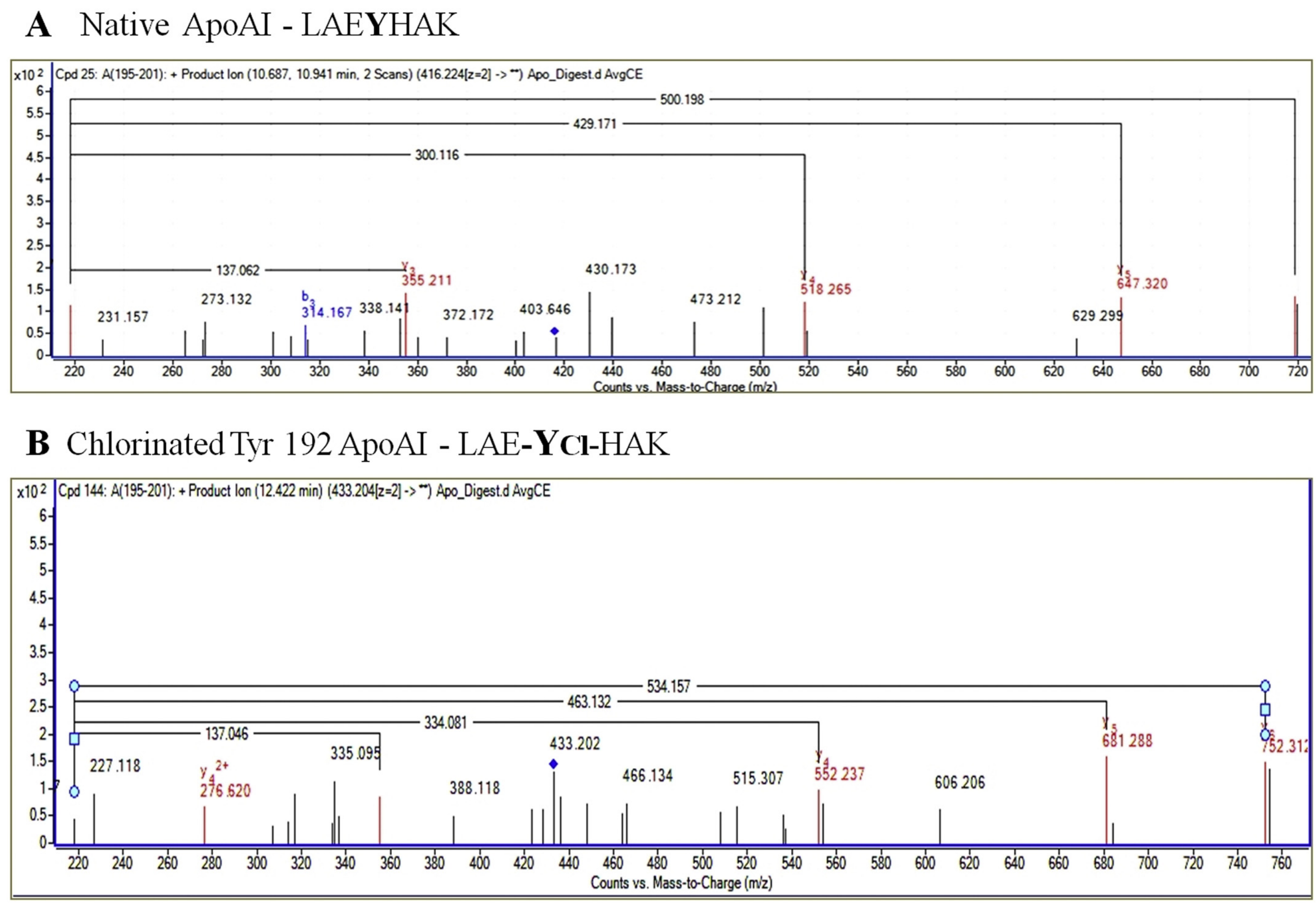

Fig. 2. Mass spectrometric analysis of native and chlorinated ^{192} tyrosine apoA-I protein by ESI-LC-MS/MS. ApoA-I (5 µg) was exposed to sodium hypochlorite for 20 min at 4 °C in phosphate buffer and the reaction was terminated with 20 mM methionine (See Section 2.6). Later, the native and chlorinated apoA-I was digested with trypsin in100mM NH4HCO3, pH 8. Digestion was halted by acidification (pH 2–3) with trifluoroacetic acid and the peptides were analyzed with LC-ESI-MS/MS. (A) Native apoA-I, the peaks which represents the molecular mass of y ions and indicate the mass of native apoA-I (830[M + H] +) (B) Reaction of sodium hypochlorite with native apoA-I generated chlorinated ^{192} tyrosine apoA-I protein which shows molecular mass at (865[M + H] +), which confirms the presence of chlorine atom (atomic mass-35) at position 192 tyrosine residue.

Table 1
Mass spectrometric analysis of trypsinized native apoA-I and chemical chlorinated ^{192} tyrosine apoA-I.

	Native apoA-I	Chemically chlorinated ^{192} tyrosine apoA-I
Sequence	L A E –Y– H A K y7 y6 y5 y4 y3 y2 y1	L A *E*- Y_{Cl}- H A K y7 y6 y5 y4 y3 y2 y1
Molecular mass	830[M + H]$^{+}$ 415[M + 2H]$^{2+}$	865[M + H]+ 433 [M + 2H]2+
Ions	Native apoA-I mass	Chemically chlorinated ^{192} tyrosine apoA-I
Y2	218	218
Y3	355	355
Y4	518	552
Y5	647	681
Y6	718	752

chemically chlorinated apoA-I protein, BSA conjugated chlorinated and non chlorinated apoA-I peptides were used for screening the hybridomas after fusion of Sp2/0 myeloma cells with spleen cells from immunized BALB/c mice. The stable clones, *viz.* F7D5 (obtained from mouse immunized with chlorinated ^{192} tyrosine full length apoA-I protein) and G11E3 (obtained from mouse immunized with synthetic chlorinated ^{192} tyrosine apoA-I peptide) producing monoclonal antibodies were obtained after several screenings and were used for further for purification and characterization studies.

3.7. Purification of monoclonal antibodies using MEP (4-Mercapto-Ethyl-Pyridine) HyperCel column

MEP HyperCel is a high capacity, high purity and high selectivity sorbent specially designed for the capture and purification of monoclonal and polyclonal antibodies. In our experiment, we loaded the cell culture supernatant directly onto the column after equilibration with 50 mM PBS (pH 7.4) without adjustment of pH or ionic strength. The MEP HyperCel adsorption is based on mild hydrophobic interaction which is achieved without the addition of other salts. Desorption is based on charge repulsion by reducing the pH of the buffer. For F7D5 clone, the flow through (PBS pH 7.4) or non-bound fraction contained albumin predominantly and the fraction eluted at pH 5.0 contained the pure antibody when compared to fractions eluted at pH 4.0 and pH 3.0 which had other unwanted proteins. For G11E3 clone, the flow through was carried out with PBS pH 7.4 and the column subsequently washed with 0.1 mM sodium acetate pH 5.5 with 0.5 M NaCl. The pure antibody was eluted with 0.1 M sodium acetate pH 3 and other unwanted proteins were come out in 0.05 mM Sodium acetate pH 4.8.

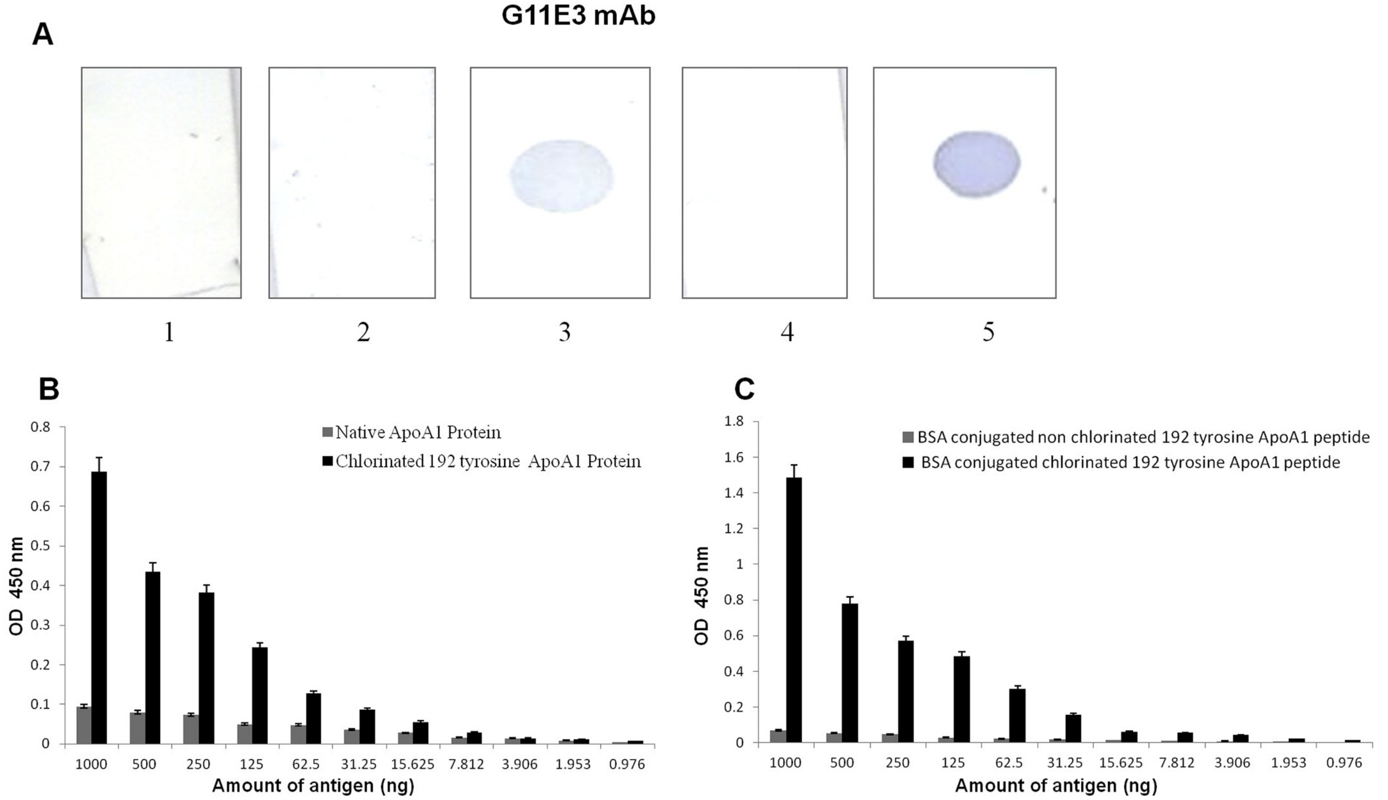

Fig. 3. Characterization of G11E3 mAb. 100 µg KLH conjugated chlorinated 192 tyrosine apoA-I peptide was used as antigen for mice immunization. After obtaining sufficient titers, hybridomas were generated as described (See Section 2.9). Subsequently the hybridomas secreting specific mAbs were screened and selected against the target antigen. (A) Specificity of the purified G11E3 mAb was determined by dot blot analysis. The reactivity of the G11E3 mAb with 3 µg of the following antigens was revealed with chromogenic substrate (See Section 2.11.1). Lane 1- control (without antigen), Lane 2 – Native apoA-I, Lane 3 –Chlorinated 192 tyrosine apoA-I protein, Lane 4 –BSA conjugate non chlorinated 192 tyrosine apoA-I peptide, Lane 5- BSA conjugate chlorinated 192 tyrosine apoA-I peptide. (B) Antigen sensitivity was determined by indirect ELISA method. Antigens were serially diluted starting from 1000 ng to 0.9 ng per well and probed with the primary antibody (G11E3 mAb) diluted (1 µg /0.5 mL). The native and chlorinated 192 tyrosine apoA-I protein were tested as antigens and (C) BSA conjugated non chlorinated 192 tyrosine apoA-I peptide and BSA conjugated chlorinated 192 tyrosine apoA-I peptide were also used as antigens.

3.8. Characterization of monoclonal antibodies

3.8.1. Isotyping

Isotyping of the selected antibodies showed that the clone F7D5 mAb belonged to the subclass IgG3 and G11E3 mAb to the subclass IgG2b. Both light chains were of the kappa isotype.

3.8.2. Dot blot analysis and antigen sensitivity

In the dot blot analysis, (Fig. 3A) G11E3 mAb showed reactivity only to chlorotyrosine containing apoA-I full-length protein or apoA-I peptide but not to native apoA-I full-length protein or native peptide without chlorotyrosine. F7D5 mab (Fig. 4A), also displayed reactivity with chlorinated 192 tyrosine apoA-I protein and apoA-I peptide. It also stained native apoA-I protein with lower intensity but did not stained native apoA-I peptide.

The two stable selected clones were characterized for the antigen sensitivity (Figs. 3B, C, 4B and C). The results showed that both mAbs (G11E3 and F7D5) showed better sensitivity (down to 31.25 ng and 15.625 respectively) for both the chlorinated 192 tyrosine apoA-I protein and synthetic chlorinated 192 tyrosine apoA-I peptide compared to non-oxidized forms. The G11E3 mAb showed less background sensitivity than the F7D5 mAb to the non-chlorinated apoA-I or non-chlorinated apoA-I derived peptide.

3.8.3. Cross reactivity

The cross reactivity of the selected two mAbs was evaluated against the two types of oxidation (chloro or nitro) and against the position of oxidized tyrosine (192 or 166). The two carrier proteins (KLH and OVA) conjugated with different peptides were commercially bought. Here the KLH-chloro peptide was used as an immunizing antigen, and the reactivity was checked with different conjugates *viz.*, BSA and OVA-chloro peptide at different positions of apoA-I residue. For this reason, we evaluated the mAbs with BSA and OVA- conjugated Chloro- apoA-I peptide in order to obtain mAbs bioreactive towards chloro- 192 tyrosine apoA-I only.

We tested G11E3 and F7D5 mAbs with four different modified apoA-I peptides containing chlorination and nitration of tyrosine residues at sites 192 and 166 respectively. Antigens were serially diluted ranging from 1000 ng to 0.9 ng per well and probed with the primary antibody (2 µg /ml) for their sensitivity. (Fig. 5A and B) represent G11E3 mAb and F7D5 mAb respectively tested with the four modified peptides.

The antigen sensitivity curve showed that the G11E3 mAb reacted to both chloro and nitro-tyrosine (position 192) containing apoA-I peptide. But we observed that still at the antigen concentration of 62.5 ng, the OD values were higher (double) for the mAbs reactivity with KLH chloro 192 tyrosine than with the KLH nitro 192 tyrosine. On the other hand no reactivity was observed against oxidized 166 tyrosine whatever oxidation type (chloro or nitro). Interestingly the F7D5 mAb showed reactivity only to chloro 192tyrosine apoA-I and not to other modified peptides of apoA-I peptide (neither nitro 192 tyrosine nor chloro/nitro 166 tyrosine).

3.8.4. Assessment of monoclonal bioreactivity on biological samples by immunohistochemistry

As the final aim of our study is to develop diagnostic tools based on

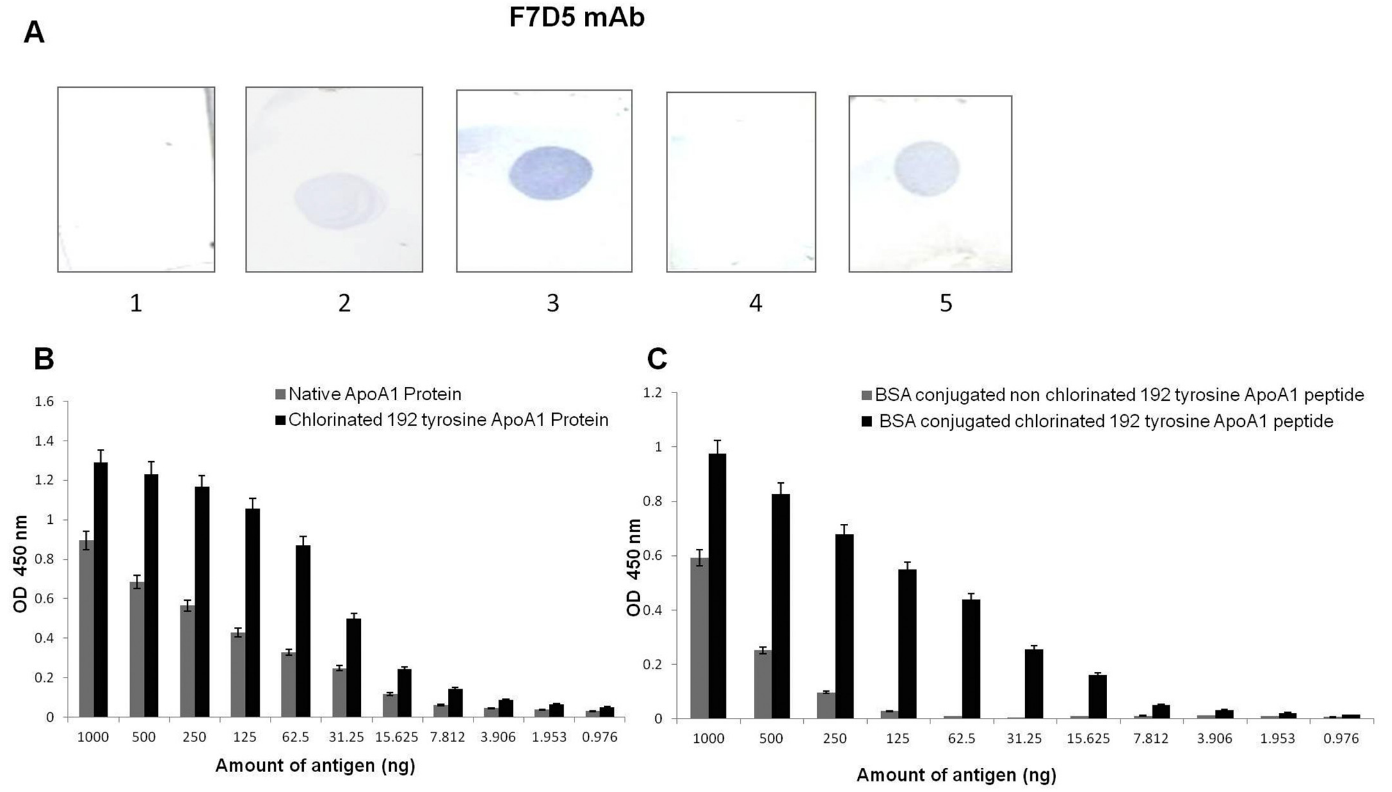

Fig. 4. Characterization of F7D5 mAb. Here 100 μg of chlorinated ^{192} tyrosine apoA-I protein was used as an antigen for immunization. After obtaining sufficient titers, hybridomas were generated as described (See Section 2.9). Subsequently the hybridomas secreting specific mAbs were screened and selected against the target antigen. (A) Dot blot analysis for purified F7D5 mAb against the following antigens: Lane 1- blank (without antigen), Lane 2 – Native apoA-I, Lane 3 –chlorinated ^{192} tyrosine apoA-I protein, Lane 4- BSA conjugated non chlorinated ^{192} tyrosine apoA-I peptide, Lane 5 –BSA conjugated chlorinated ^{192} tyrosine apoA-I peptide. (B) Antigen sensitivity was determined by indirect ELISA method. Antigens were serially diluted starting from 1000 ng to 0.9 ng per well and probed with the primary antibody (F7D5 mAb) diluted (1 μg /0.5 mL) for their sensitivity. The native and chlorinated ^{192} tyrosine apoA-I protein were tested as antigens and (C) as mentioned above, BSA conjugated non chlorinated ^{192} tyrosine apoA-I peptide and BSA conjugated chlorinated ^{192} tyrosine apoA-I peptide were also used as antigens.

antibodies detecting chlorinated apoA-I for predicting CVD risks in humans, we evaluated the ability of F7D5 and G11E3 antibodies to recognize ^{192}tyrosine apoA-I within aorta lesional sections of animal models of atherosclerosis as well as aorta sections recovered from human endarterectomies (Fig. 6).

Arterial sections from ApoE − / − mice showed a strong labelling of the intima in atheroma with both antibodies. The same strong labelling was observed when sections of human coronary specimens were incubated either with F7D5 or G11E3 antibodies. Enlarged views showed the staining of necrotic area located within the disorganized intima for ApoE − / − mice and human arterial sections for both antibodies. No labelling was observed with controls performed with secondary antibody. We can observe that the labeling with the G11E3 antibody on mouse or human arterial specimens is intensively distributed both within the disorganized areas of the intima, and in the media and adventitia in comparison with the staining of F7D5 antibody restricted to the area of the intima. These results may be in accordance with the fact that G11E3 clone may recognize chloro ^{192} tyrosine apoA-I protein but also to a lesser extent nitro ^{192} tyrosine apoA-I peptide in atheroma plaque biopsies of both species, whereas F7D5 antibody preferentially recognizes chloro ^{192} tyrosine apoA-I protein only. These immunohistochemistry data were in accordance with antigen sensitivity and cross reactivity assays with F7D5 or G11E3 antibodies, showed in (Figs. 3, 4 and 5).

4. Discussion

This work describes the expression of recombinant apoA-I using bacterial expression system and optimizes the condition for chemical chlorination of ^{192} tyrosine apoA-I. Subsequently, we developed mAbs against both chemically chlorinated ^{192} tyrosine apoA-I and synthetic chlorinated ^{192} tyrosine apoA-I peptide. It is reported that apoA-I -HDL, which was considered as cardio protective, undergoing oxidation at a number of sites by myeloperoxidase, not only renders apoA-I dysfunctional, but also converts apoA-I -HDL into a proinflammatory and proatherogenic state (Shao et al., 2010). Several studies indicated that myeloperoxidase-mediated apoA-I oxidation impairs the cholesterol efflux from macrophage foam cells in the human artery wall. Oxidized apoA-I has been found in atherosclerotic plaque and blood; the level of both 3-chlorotyrosine and 3-nitrotyrosine was higher in coronary artery disease patients than healthy individuals, which strongly implicates myeloperoxidase as a key indicator to HDL oxidation *in vivo* (Pennathur et al., 2004; Bergt et al., 2004a, 2004b; Shao et al., 2012). Thus, the chlorination of apoA-I may play a major role in inhibiting functional HDL and reverse cholesterol transport during atherogenesis. A number of studies have suggested that the oxidized apoA-I in circulation may serve as a biomarker of cardiovascular risks in humans. It is observed that the apoA-I from atherosclerotic plaque could either be chlorinated or nitrated at position 166 and 192 tyrosine as they are one of the most susceptible sites of oxidized modification by myeloperoxidase (DiDonato et al., 2014; Bergt et al., 2004a, 2004b). Most of the studies have characterized the levels of oxidized apoA-I in atherosclerotic plaques but few studies in the circulation and only by proteomics analysis, which cannot be used routinely in clinical settings. Based on the reported data, we initiated our research work, in order to develop mAbs specific against the chlorinated ^{192} tyrosine apoA-I.

In our study, the recombinant apoA-I was expressed as a fusion protein with glutathione S- transferees (GST) tag, which permits one-

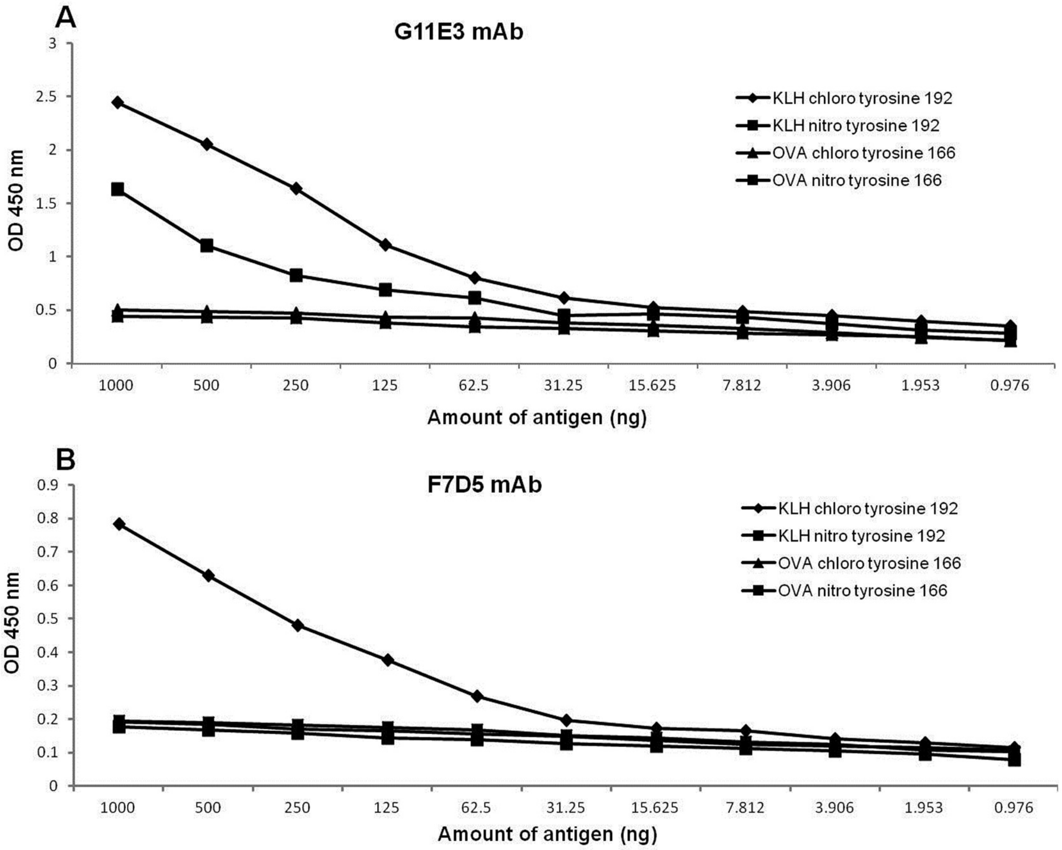

Fig. 5. Both G11E3 mAb (generated against the chlorinated peptide of apoA-I) and F7D5 mAb (generated against chlorinated apoA-I full length polypeptide) were highly specific to apoA-I peptides (189-LAE-3-YCl-HAK -195) containing chlorinated ^{192} tyrosine. Cross reactivity of mAbs with apoA-I peptides containing nitrated or chlorinated tyrosine residues at 192 and 166 in the sequence were analyzed as described in methods (See Section 2.11.4).

Specificity of G11E3 mAb (A) and F7D5 mAb (B) is shown. The four different modified apoA-I peptides were serially diluted starting from 1000 ng to 0.9 ng and probed with the primary purified antibody mAb after dilution (1 μg /0.5 mL) and indirect ELISA was carried out. Absorbance was monitored at 450 nm and antigen sensitivity graph was plotted.

step purification of fusion protein from crude bacterial cell lysates using the GST affinity system. Once purified, the GST tag was removed from apoA-I by using thrombin and the purified recombinant apoA-I was taken to optimize the condition for the chemical chlorination. The chlorination of tyrosine at position 192 was determined using mass spectrometer and the modified apoA-I was further taken for specific monoclonal antibody development. Along with this modified recombinant protein, a synthetic peptide containing chlorinated tyrosine, which represents position 192 in apoA-I, was also used for generating monoclonal antibodies. Two monoclonal antibodies (F7D5 and G11E3) with high bioreactivity and sensitivity were obtained.

The F7D5 monoclonal antibody developed against the chlorinated recombinant apoA-I protein showed better reactivity towards chlorinated apoA-I protein and synthetic chlorinated tyrosine apoA-I peptide compared to non-chlorinated tyrosine apoA-I peptide. The G11E3 monoclonal antibody developed against synthetic chlorinated ^{192}tyrosine apoA-I peptide showed specificity towards synthetic chlorinated ^{192} tyrosine apoA-I peptide and, chlorinated recombinant apoA-I protein with neither recognition of the non-chlorinated tyrosine apoA-I peptide nor native apoA-I protein. Both mAbs were also tested against other peptides with chlorination and nitration modifications at 192 and 166 amino acid residue sites in apoA-I for further determining their bioreactivity towards respective antigen. These observations strongly indicate that the F7D5 mAb developed against chlorinated apoA-I protein was highly reactive to apoA-I peptide containing chlorinated ^{192} tyrosine and that G11E3 mAb developed against the chlorinated peptide of apoA-I also recognizes the peptide with a nitrated tyrosine residue at 192 but at least two times less than the apoA-I peptide containing chlorinated ^{192} tyrosine.

Interesting results were obtained in IHC analyses on slices of human atheroma plaques from endarteriectomies and ApoE−/− animal model of atherosclerosis. G11E3 mAb developed against the chlorinated peptide of apoA-I and cross-reacting with the nitrated peptide, exhibited a strong reactivity in the intima of the atheroma plaques and even in the media of the human plaques. F7D5 mAb developed against chlorinated apoA-I protein, even if it presented a faint cross-reactivity with apoA-I native protein, showed a more localized specificity in the necrotic core of the intima, both in human and mouse atheroma sections.

Currently, mass spectrometric methods are available to detect the chlorination of apoA-I however they cannot be adopted in clinical settings in high throughput assays as they are time consuming, expensive and requires well trained manpower to maintain the sophisticated facility. To overcome these difficulties, immune based assays serve the purpose to predict CVD risks in human. Therefore the development of mAbs against chlorinated apoA-I is absolutely essential for predicting CVD risks in large number of clinical samples and the developed monoclonal antibodies can provide a diagnostic tool for predicting CVD risks in humans since oxidation of HDL seems to occur much earlier than the disease itself and the circulating HDL- apoA-I may serve as an early biomarker of CVD risks.

To conclude, here we have developed monoclonal antibodies F7D5 and G11E3 which present a highly reactive towards chlorinated ^{192} tyrosine apoA-I, in order to be used robustly and routinely in the clinical settings. Specifically we have used a synthetic chlorinated peptide of apoA-I and chlorinated apoA-I as antigens to obtain these mAbs with

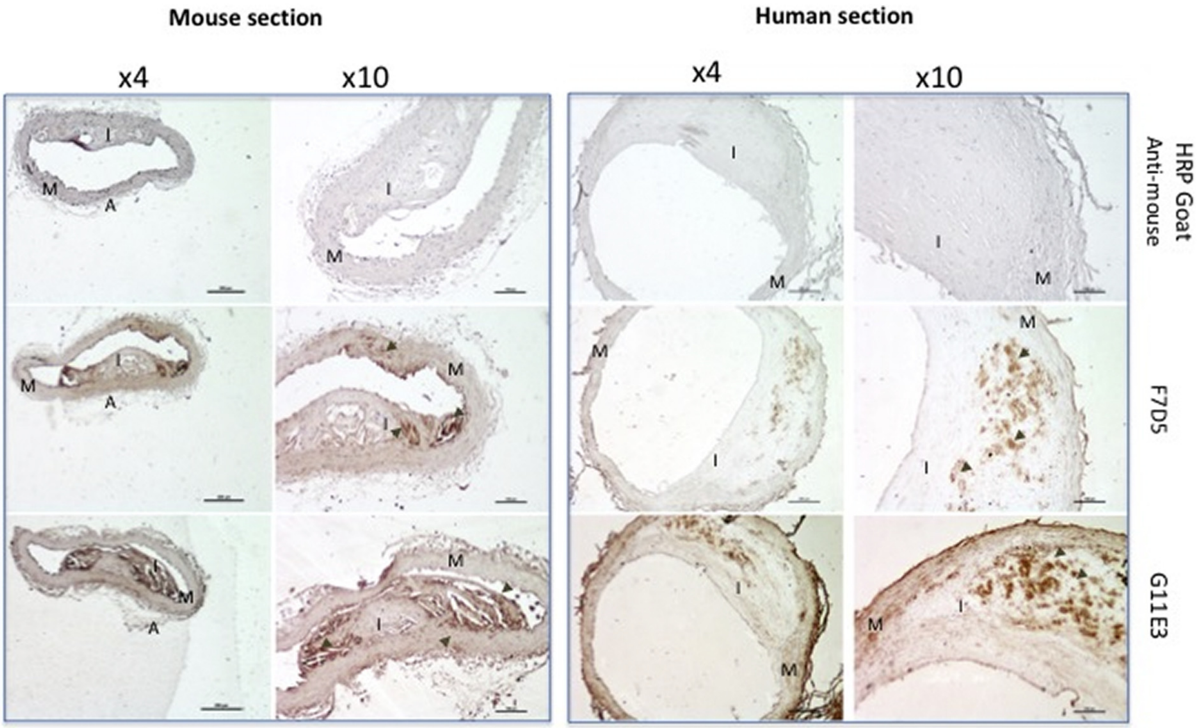

Fig. 6. Immunoreactivity of anti-chloro ^{192}tyrosine HDL- apoA-I antibodies F7D5 and G11E3 has been assessed by immunochemistry analysis on atherosclerotic mouse and human tissues. Binding of antibodies was visualized *via* HRP-anti-mouse antibody. Nuclei were counterstained with hematoxylin. Lane 1: Negative control was secondary antibody only. Lane 2 and 3: F7D5 and G11E3 antibodies specifically recognized the injured areas of the aorta sections from mouse and human species, particularly in the intima (black arrows on enlarged views). Bar: 250 μm and 100 μm for 4× and 10× magnifications, respectively.

the aim to subsequently develop ELISA based methods for detecting the extent of chlorinated ^{192} tyrosine of apoA-I in myocardial infarction patients. Moreover, the fact that both mAbs are able to stain mouse and human atheroma in IHC experiments is of high interest for *in vivo* atherosclerosis imaging, allowing translation from pre-clinical to clinical studies. A successful development of these anti-chloro- ^{192} tyrosine apoA-I antibodies may pave the way for their use in both early diagnosis and prognosis.

Declaration of Competing Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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