Original Research

Activity of MUC1 cancer antigen-binding plasma anti-α-galactoside antibody correlates inversely with size of autologous lipoprotein(a)

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Impact statement

Lower cancer incidence in higher plasma titer/smaller size Lp(a) individuals remains unexplained. We show that specific activity of anti-Gal, the prominent natural antibody against cancer-specific MUC1 antigen, varied inversely with the size of Lp(a) which also bears serine- and threonine-rich peptide sequences (STPS) recognized by anti-Gal. With increasing Lp(a) size, the increasing number of LDL molecules noncovalently adhering to it progressively decreased the accessibility of STPS. Resulting variations in antigenicity of Lp(a) could modulate anti-Gal activity possibly by affinity maturation since Lp(a) has lipid component and immunoglobulin class switching of anti-Gal is more in smaller Lp (a) individuals. Besides explaining the modulation of activity of the dominant primate-specific anti-cancer antibody by size polymorphism of an endogenous primate-specific macromolecular antigen, the data offer infusion of high activity anti-Gal or small Lp(a) as therapeutic option in cancer. Our subsequent clinical study confirmed the negative correlation of anti-Gal activity with breast cancer incidence.

Abstract

Variation in ligand-binding affinity of natural plasma anti-α-galactoside antibody (anti-Gal) is a plausible reason for differing anti-cancer defense among individuals since serine- and threonine-rich peptide sequences (STPS) in the cancer-specific MUC-1 antigen are surrogate ligands for this antibody. As affinity of a natural antibody could be modulated by systemic antigens by processes including affinity maturation, we examined the contribution of the size of lipoprotein(a) [Lp(a)], an efficient autologous anti-Gal-binding macromolecule that possesses variable numbers of STPS due to genetically determined size polymorphism, towards the specific activity (activity per unit mass) of anti-Gal. Binding of purified Lp(a) to FITC-labeled anti-Gal, measured in terms of increase in fluorescence of the latter, was inhibited by LDL in proportion to Lp(a) size presumably because LDL molecules also bind noncovalently and in proportion to Lp(a) size at the O-glycosylated and STPS-rich region of Lp(a). For the same reason, circulating forms of smaller Lp(a) which carried fewer or no noncovalently attached LDL molecules were more efficient ligands for the antibody than the same number of larger ones ($P < 0.0001$). Result suggested that smaller Lp(a), with their STPS ligands less obstructed by adhering LDL, would be more effective systemic antigens for anti-Gal. In confirmation of this, the specific activity of anti-Gal decreased with Lp(a) size ($r - 0.5443$; $P < 0.0001$) but increased with Lp(a) concentration (r 0.6202; $P < 0.0001$) among 73 normal plasma samples. IgG to IgM ratio, an index of immunoglobulin class switching characteristic of affinity maturation, was decidedly higher for anti-Gal in small Lp(a) individuals than in their large Lp(a) counterparts ($P = 0.0014$). Results indicated that modulation of activity of anti-Gal by Lp(a) size may account for the lower incidence of cancer reported in people carrying more plasma Lp(a) which are generally smaller as well.

Keywords: Lipoprotein(a), Lp(a) size polymorphism, anti-α-galactoside antibody, serine-and threonine-rich peptide sequence, affinity maturation, cancer susceptibility

Experimental Biology and Medicine **2019; 244: 893–900. DOI: 10.1177/1535370219855002**

Introduction

Overexpression of the serine-and threonine-rich peptide MUC1 with or without glycosylation at these residues is a hallmark of cancer cells[1] so that vaccination for increased production of anti-MUC1 antibodies is a widely pursued therapeutic strategy.[2] Naturally occurring anti-α-galactoside antibody (anti-Gal) present in plasma of primates had been shown to recognize MUC1 as surrogate ligand by accommodating the serine-and threonine-rich peptide sequence (STPS) of MUC1 at its binding site.[3]

Though these observations suggested that variations in specific activity (ligand-binding activity per unit immunoglobulin protein) of anti-Gal would be decisive in natural anti-tumor defense, the factors affecting specific activity of this antibody are hardly known. Circulating lipoprotein(a) [Lp(a)] which is also exclusive to primates has been found to form immune complex with anti-Gal.[4] The ligand recognized by anti-Gal on Lp(a) is also the STPS underlying the profuse O-glycans in the latter's apo(a) subunit since de-O-glycosylation of apo(a) only increased anti-Gal binding.[4] Molecular weight of Lp(a), that depends on the genetically determined number of repeats of its kringle IV type 2 domain, varies widely among individuals.[5] High plasma concentration of Lp(a)which is generally accompanied by smaller size of the lipoprotein,[6] correlates positively with atherosclerosis, stroke, and neurodegenerative disorders.[7] Curiously, low plasma Lp(a) concentration which usually occurs in large Lp(a) individuals is associated with increased incidence of cancer[8] though no causative relationship has been established.

Persistent presence in the host of systemic molecules possessing antigenic ligands is known to effect affinity maturation of antibodies resulting in antibody molecules with greater complementarity and affinity to the systemic antigenic ligand.[9] While synthesis of anti-Gal has been suggested to be triggered by gut microbial antigens,[10] presence of anti-Gal:Lp(a) immune complex in plasma[4] underlined the potential of Lp(a) as an autologous antigenic effector of affinity maturation of this antibody. A major determinant of availability of STPS in Lp(a) for recognition by anti-Gal is likely to be the number of LDL molecules that noncovalently adhere to the circulating form of the lipoprotein in addition to its integral LDL molecule to which the apo(a) subunit is covalently linked. Reason is that adhering or adduct LDL molecules utilize the sialic acid-rich and negatively charged O-glycosylated STPS-containing region of apo(a) for charge-dependent binding resulting in blocking of access of anti-Gal to Lp(a). Since marked increase in the number of adduct LDL molecules with increasing Lp(a) size had been reported,[11] smaller Lp(a) molecules with little or no adduct LDL would have their STPS-containing region less obstructed by LDL and more exposed in order to get better recognized by anti-Gal antibody to act as efficient affinity maturation antigens. In this communication, we investigated how individual variations in size and plasma concentration of Lp(a) affected the specific activity of anti-Gal since the latter property may decide the individual's antibody-mediated resistance to cancer.

Materials and methods

Materials

Methyl-α-D-galactopyranoside, galactose, melibiose, cellobiose, soybean trypsin inhibitor, soluble guar gum, horse radish peroxidase (HRP) type II, ortho-phenylenediamine (OPD), sodium cyanoborohydride, dithiothreitol (DTT), fluorescein isothiocyanate (FITC), Tween-20, and hydrogen peroxide were purchased from Sigma-Aldrich, Bangalore, India. Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden. Polystyrene 96-well break-apart microplates (MAXISORB) were purchased from Nunc, Denmark. Antibodies to human IgA, IgG and IgM raised in goat and antibodies to apo(a) and apoB raised in rabbit were purchased from Dako, Denmark. Lp(a) assay standard (99 mg/dL; International Reference SRM 2B Standardization) was obtained from APTEC Diagnostics nv, Belgium) All other chemicals used were of analytical grade. The seeds of *Artocarpus integrifolia* (jack fruit) were obtained locally. Outdated plasma samples from healthy individuals of age 18–40 years were collected from the Department of Blood Transfusion Services of this Institute with Institutional Ethics Committee approval (SCT/IEC/926).

The affinity chromatography matrix cross linked guar galactomannan (CLGG) was prepared by cross linking soluble guar galactomannan (from *Cyamopsis tetragonolobus* beans) using epichlorohydrin to form an insoluble gel by a procedure described earlier.[12] The lectin, jacalin was prepared from the seeds of *Artocarpus integrifolia* (jack fruit) by affinity chromatography on CLGG.[13] Jacalin-Sepharose 4B matrix was prepared by coupling jacalin to Sepharose 4B using cyanogen bromide.[14] Antibodies against apo(a), apoB, human IgA, IgG, IgM were labeled with HRP as described earlier.[15] Melibiose or cellobiose was covalently coupled to the non-glycosylated protein soybean trypsin inhibitor by reductive amination using sodium cyanoborohydride[16] to obtain neoglycoconjugates TIM or TIC. Soluble proteins were assayed by Bradford method using bovine serum albumin as standard.[17]

Isolation of antibodies

Anti-Gal and anti-β-glucan antibody (ABG) specific to β-glucosides were isolated from outdated human plasma samples. Anti-Gal has been found to form immune complexes with lipoprotein(a)[4] as well as two albumin-bound O-glycosylated proteins.[18] Treatment with specific sugar dissociated these complexes and subsequent ultracentrifugation led to sequestration of ligand-free anti-Gal to the bottom. Plasma (70 mL) was treated with 0.2 M galactose overnight at 4°C and subjected to ultracentrifugation at 202,000*g* in 1.24 g/mL density at 4°C for 4 h in 4 mL tubes. Bottom 30% volume from all tubes were pooled, dialyzed against 20 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS) and loaded on CLGG column for affinity chromatography. After washing with PBS to remove unbound proteins, bound anti-Gal was eluted with 150 mM galactose in PBS. ABG from human plasma was isolated by affinity chromatography on cellulose-celite (1:1 volume) column.[19] Anti-Gal purified by affinity chromatography was labeled with FITC as described by Hudson and Hay.[20]

Lp(a) determination by jacalin-based enzyme immunoassay

Lp(a) in plasma and other lipoprotein samples was determined by an apoB-independent jacalin-based enzyme immunoassay.[21] Briefly, polystyrene wells coated with jacalin (1 μg in 200 μL PBS) by incubating at 37°C for 3 h were washed with PBS containing 0.05% Tween-20 (PBST) and

blocked with PBS containing 0.5% Tween-20 at 37°C for 30 min. After washing again with 0.05% PBST, wells were incubated with 500 times diluted plasma or appropriate dilution of protein sample in 200 μL 0.05% PBST for 2 h at 4°C. Washed wells were probed with 200 μL HRP-conjugated anti-human apo(a) [1.5 μg per mL] in 0.05% PBST. After washing, bound HRP was assayed by treating wells with 200 μL OPD (0.5 mg/mL) in 0.1 M citrate phosphate buffer, pH 5.0 containing 0.03% H_2O_2 for 15 min, followed by stopping the reaction with 50 μL of 12.5% H_2SO_4 and absorbance was read at 490 nm in ELISA reader (BioTek USA).

Isolation of Lp(a) from human plasma

Lp(a) was prepared by affinity precipitation of plasma proteins with lectin jacalin followed by electrophoresis and elution of lipoprotein bands as described earlier.[22] Briefly, proteins precipitated from normal plasma by jacalin were re-dissolved using lectin-specific sugar and subjected to ultracentrifugation, whereby natural form of Lp(a) with adduct LDL bound to it (L1) sequestered in the top 20% volume. Pure Lp(a) and LDL were separated by electrophoresis of L1 in Tris-Borate-EDTA buffer at pH 8.7 and recovered by passive elution to PBS.

Molecular size index of Lp(a) isoforms

Appropriate dilution of eluted Lp(a) isoforms from different individuals was directly coated on microtiter plate wells. After blocking and washing, wells were separately probed with HRP-labeled anti-human apo(a) and anti-human apoB (1.5 μg per mL of each antibody) and the bound HRP activity was assayed as described for Lp(a) assay above. The ratio of absorbance for anti-apo(a), [a] to that for anti-apoB, [B] at 490 nm ([a]/[B]) was taken as an index of molecular size of Lp(a).

Preparation of apo(a)

Apo(a) was prepared by DTT reduction of plasma lipoproteins by a method described earlier.[4] Plasma (1 mL) was subjected to ultracentrifugation at 535,000*g* for 4 h at 4°C after adjusting the density to 1.24 g/mL with potassium bromide and top 20% layer (L1) containing lipoproteins was collected and dialyzed against PBS. This sample was subjected to reduction using 4 mM DTT at 37°C for 15 min. After adjusting density of the solution to 1.24 g/mL with potassium bromide, the solution was again subjected to ultracentrifugation at 535,000 g for 4 h at 4°C. Bottom 20% layer containing apo(a) alone[4]was collected and dialyzed against PBS.

Preparation of O-glycan-rich glycoproteins other than apo(a) from plasma

After preparation of L1 from 2 mL plasma as described above, the non-L1 plasma proteins (bottom 80% of plasma after ultracentrifugation) were collected, dialyzed against PBS, and loaded to jacalin-Sepharose 4B affinity chromatography column (5 mL). Unbound protein was washed out with PBS and bound protein eluted with 0.8 M galactose in PBS. Eluted proteins were collected, concentrated, and subjected to 6% alkaline PAGE electrophoresis. After staining, different bands of O-glycan-rich glycoproteins were passively eluted as described above for Lp(a) and protein estimated in each sample.

Plasma anti-Gal IgG and IgM levels

Polystyrene wells were coated with melibiose-conjugated soybean trypsin inhibitor (TIM, 1 μg/well) and blocked as described. Plasma diluted 50× was added and wells were incubated for 2 h at 4°C. After washing, wells were probed with 200 μL HRP-conjugated anti-human IgG and IgM separately (1.5 μg/mL in PBST) for 2 h at 4°C, washed again and bound HRP was assayed using OPD as substrate as described above for Lp(a) assay.

Determination of specific activity of anti-Gal and ABG antibody

Specific activity of anti-Gal and ABG was defined as the ratio of ligand-binding activity to immunoglobulin content of the same amount of antibody.[23] Binding to TIM and TIC was used as indicator of activity of anti-Gal and ABG, respectively. To measure ligand-binding activity, polystyrene wells were coated with TIM or TIC (1 μg per well in 200 μL PBS) and after blocking, wells were incubated with 50 ng anti-Gal or 250 ng ABG in 200 μL PBST and washed. To assay immunoglobulin content, microwells coated with anti-Gal or ABG (50 ng in 200 μL PBS) were blocked and washed. Bound antibody in all the above wells was assayed by probing with a mixture of HRP conjugates of anti-human IgG, IgM, and IgA (1.5 μg per mL PBST of each antibody; 200 μL per well) and bound HRP activity assayed as described. Ratio of ligand binding assay response and immunoglobulin assay response of 50 ng antibody represented specific activity.

Fluorometric assay of ligand binding to FITC-anti-Gal

FITC-anti-Gal incubated alone or with defined amounts of ligands in 75–100 μL PBS for 18 h at 4°C was brought to 300 μL in PBS and fluorescence measured using excitation at 485 nm and emission at 525 nm in a BIOTEK FLx800 fluorescence reader employing a sensitivity level of 50 throughout.

Statistical analysis

All results were expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison tests or unpaired Student's t-test was used to determine the significance of difference between the groups where appropriate. Pearson's correlation coefficient, r was used to measure the strength and the direction of a linear relationship between two parameters. A value of $P < 0.05$ was considered statistically significant. Data were statistically analyzed using GraphPad Prism version 5.0 software.

Results

Noncovalently attached LDL blocks recognition of Lp(a) by anti-Gal

Since in general plasma Lp(a) concentration is inversely proportional to the size of the individual's Lp(a),[6] the former parameter which is more easily measurable was used as indicator of Lp(a) size. Since Lp(a) concentrations between 10 mg/dL and 30 mg/dL are generally considered normal, plasma samples containing Lp(a) concentrations within this range were chosen for preparation of purified (free from adduct LDL) Lp(a) of average size. Affinity of Lp (a) as a ligand for anti-Gal was measured in terms of the percentage of increase in fluorescence of fluorescently labeled anti-Gal. This increase had been reported to be a measure of conformational shift in the Fc part of the antibody which in turn reflected the strength of antigen binding at the binding site since low molecular weight ligands that produced insignificant conformational changes in the Fc part and non-reactive macromolecules produced little or no increase in the fluorescence of fluorescently labeled antibody.[23] Result in Figure 1 showed that the significant increase in the fluorescence of labeled anti-Gal brought about by pure Lp(a) was reduced to marginal if the same Lp(a) pre-treated with LDL was used (P value < 0.001). Complete abolition of Lp(a)-induced fluorescence increase by the presence of the disaccharide anti-Gal ligand melibiose confirmed that Lp(a) occupied the sugar binding site of anti-Gal. Added LDL either competed with anti-Gal for binding to Lp(a) or blocked binding of the antibody since specificity of anti-Gal was for Lp(a) only and LDL alone failed to enhance the fluorescence of FITC-anti-Gal. LDL noncovalently attached to Lp(a) is known to use the negative charges on the O-glycans of the latter to form adducts.[11] Therefore, results in Figure 1 lead to the conclusion that noncovalently attached adduct LDL in circulating Lp(a) molecules, being bulky molecules, deter recognition of the latter by the antibody possibly by blocking the O-glycan-rich region and thereby the STPS.

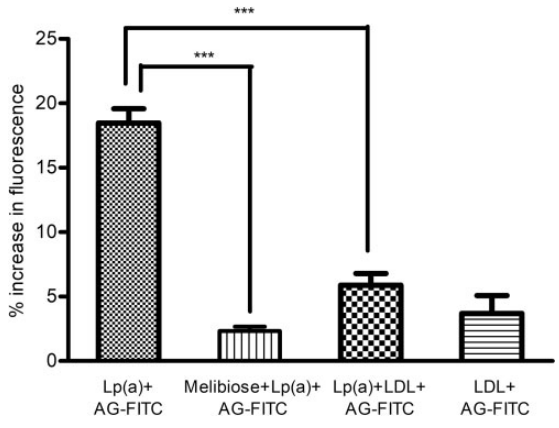

Figure 1. Inhibition of anti-Gal binding to Lp(a) by non-covalently attached LDL. Purified Lp(a) (4 μg in 50 μL PBS) was incubated with LDL (2 μg in 25 μL PBS) or with 25 μL PBS alone for 6 h at 4°C. These samples or LDL (2 μg in 75 μL PBS) were further incubated with anti-Gal-FITC (3 μg in 25 μL PBS) for 18 h at 4°C. Alternatively, anti-Gal-FITC (3 μg in 25 μL PBS) pre-incubated with its specific sugar (25 mM melibiose) in PBS for 1 h at 4°C was incubated with purified Lp(a) (4 μg in 75 μL PBS) for 18 h at 4°C. Volume in each case was made up to 300 μL with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm employing sensitivity level 50. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on addition of other ligands. Mean ± SEM of six consecutive plasma samples as Lp(a) source. ***P value <0.001.

LDL-mediated blocking of anti-Gal binding to apo(a) increases with apo(a) size

Purified samples of apo(a) subunits from high and low molecular weight Lp(a) samples (plasma Lp(a) concentrations < 10 mg/dL and > 30 mg/dL, respectively) and possessing equal antigenic apo(a) content (equal to 2 μg standard Lp(a) determined by apoB-independent assay[21]) were compared for the effect of same amount of LDL on their capacity to bind anti-Gal. Result (Figure 2) showed that binding of anti-Gal to lower molecular weight apo(a) was relatively less blocked by LDL. Since apo(a) size is the sole determinant of Lp(a) size, this result indicated that in circulation, low molecular weight Lp(a) is more available for recognition by anti-Gal antibody compared to high molecular weight Lp(a).

Circulating forms of smaller Lp(a) bind to anti-Gal more strongly than those of larger Lp(a)

Circulating forms of Lp(a) was precipitated by jacalin, redissolved by addition of jacalin-specific sugar and was segregated from other proteins to top 20% layer following ultracentrifugation (methods). This sample (JL1), from plasma contained only Lp(a) which also bears adduct LDL if any.[22] JL1 samples, all possessing the same apo(a)

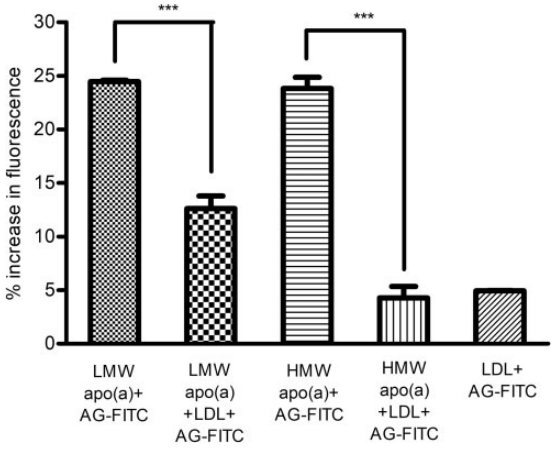

Figure 2. Inhibition of anti-Gal binding to low and high molecular weight apo(a) by LDL. To assay apo(a) content purified low molecular weight (LMW) or high molecular weight (HMW) apo(a) derived from respective Lp(a) samples (defined under 'results') was directly coated on micro plate wells (50 ng in 200 μL PBS), wells blocked and washed as described for jacalin coating in Lp(a) assay (methods). Bound apo(a) was assayed by treating with anti-apo(a)-HRP (1.5 μg antibody per mL) in 200 μL PBST, washing and assaying bound HRP as described for Lp(a) assay. LMW apo(a) (2 μg) or an amount of HMW apo(a) of equal apo(a) content based on anti-apo(a) response, in 50 μL PBS was incubated with LDL (4 μg in 25 μL PBS) or with 25 μL PBS for 6 h at 4°C. These samples or LDL (4 μg in 75 μL PBS) were further incubated with anti-Gal-FITC (3 μg in 25 μL PBS) for 18 h at 4°C. Volume was made up to 300 μL with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on addition of other ligands. Mean ± SEM of six consecutive plasma samples as apo(a) source. ***P value <0.001.

content, from low and high molecular weight Lp(a) individuals (according to criteria described for Figure 2) were treated with the same sample of FITC-labeled anti-Gal. Results in Figure 3 show that increase in fluorescence was significantly greater by JL1 of small Lp(a) individuals than by JL1 from the other group (P value < 0.0001). It followed that larger Lp(a) molecules, despite possessing more STPS-containing kringle IV type 2 repeats, were less accessible in their circulating forms to anti-Gal for binding. LDL molecules (adduct LDL) that remain adhered to circulating Lp(a) in proportion to Lp(a) size by non-covalent adhesion[11] could have blocked access of anti-Gal to the STPS in Lp(a) as happened when LDL was added to purified Lp(a) (Figure 1). Result in Figure 3 also established that qualitative differences in Lp(a) structure in circulation, and not just their number are decisive in the affinity of circulating Lp(a) for anti-Gal.

Lp(a) size and plasma concentration decide the specific activity of anti-Gal

Lp(a) of varying molecular size differs only in the size of their apo(a) chains which contain varying numbers of kringle IV type 2 repeats. Accordingly, the number of inter-kringle regions that is rich in STPS and bear O-glycans also increase with the size of apo(a) chains. In pure form with no adduct, LDL larger Lp(a) would therefore be expected to contribute more towards affinity maturation of anti-Gal. In vivo, however, the natural form in which Lp(a) occurs is occupied by noncovalently bound (adduct) LDL molecules in proportion to Lp(a) size.[11] LDL which binds to Lp(a) utilizing the negative charge on the O-glycans distributed on STPS could therefore be expected to be a confounding factor in Lp(a)-mediated effects on the activity of anti-Gal, including its affinity maturation, by restricting the accessibility of STPS, the surrogate ligand for the antibody. We examined the correlation between Lp(a) size and specific activity of anti-Gal in 73 random samples of plasma from healthy individuals. Rather than plasma concentration, a parameter which directly indicates Lp(a) size was resorted to for this purpose. Since apo(a) is attached by disulfide bond to the apoB of an LDL molecule during Lp(a) synthesis, apo(a) is peripherally available, while apoB gets masked in proportion to the size of the apo(a).[22] Consequently, anti-apoB response [B] to microplate-coated purified Lp(a) [free from adduct LDL] varied depending on apo(a) size, whereas anti-apo(a) response [a] was independent of apo(a) size, so that the ratio of responses ([a] to [B]) was an index of Lp(a) size.[22] Figure 4 shows that specific activity of anti-Gal in normal individuals varied inversely with their Lp(a) size with a correlation coefficient $r = -0.5443$ and P value <0.0001. Since size of Lp(a) synthesized varied inversely with its plasma concentration,[6] we confirmed the above effect of Lp(a) size on anti-Gal activity by determining the

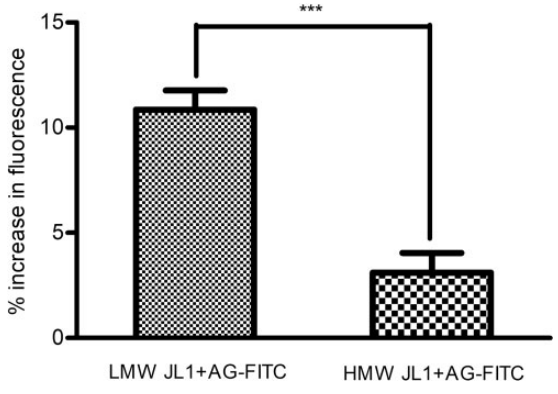

Figure 3. Recognition of circulating forms of low and high molecular weight Lp(a) samples (JL1) by anti-Gal antibody. To measure apo(a) contents of JL1 samples by ELISA, polystyrene wells were coated with jacalin (1 μg/well), blocked as described earlier and diluted JL1samples (in 200 μL PBST) were added. After incubation for 2 h at 4°C bound apo(a) was assayed by probing with anti-apo(a)-HRP (1.5 μg antibody per mL; 200 μL per well) as described for Figure 2. Low molecular weight (LMW) JL1 (6 μg) or high molecular weight (HMW) JL1 of equal apo(a) content [based on anti-apo(a) response] in 75 μL PBS was incubated with anti-Gal-FITC (3 μg in 25 μL PBS) for 18 h at 4°C. Volume was made up to 300 μL with PBS just before fluorescence measurement. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to get % increase in fluorescence on addition of other ligands. Mean ± SEM of 18 consecutive plasma samples as source of JL1 in either group. ***P value <0.0001.

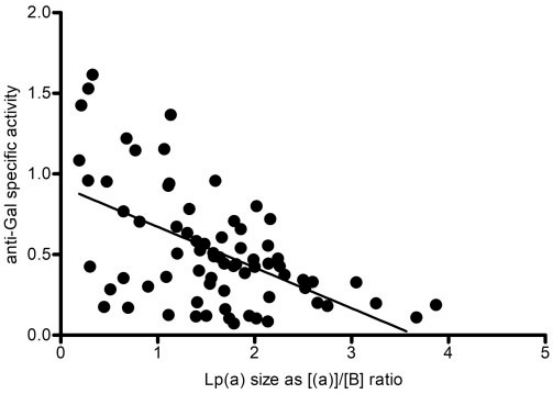

Figure 4. Correlation plot of Lp(a) size and anti-Gal specific activity. As an index of molecular size of Lp(a) ratio of anti-apo(a) response to anti-apoB response (absorbance at 490 nm) of microplate well-coated Lp(a) was measured as described in methods. Specific activity of anti-Gal antibody was determined as described in methods. $n = 73$, Pearson's correlation coefficient, $r = -0.5443$, P value <0.0001.

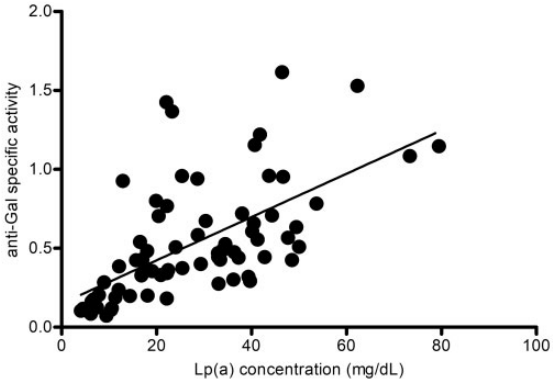

Figure 5. Correlation plot of Lp(a) concentration and anti-Gal specific activity. Lp(a) in plasma samples was assayed by an apoB-independent jacalin-based enzyme immunoassay as described in methods. Specific activity of anti-Gal antibody was determined as described in methods. $n = 73$, Pearson's correlation coefficient, $r = 0.6202$, P value <0.0001.

variation of specific activity of anti-Gal with plasma Lp(a) concentration. Since the assay used for plasma Lp(a) deciphered only the apo(a) content regardless of actual size of Lp(a), the assay values reflected the number of Lp(a) molecules irrespective of their size. Data in Figure 5 show that specific activity of anti-Gal in plasma increased with the concentration of Lp(a) with a correlation coefficient $r = 0.6202$ and P value < 0.0001.

Apo(a) is superior to other plasma O-glycosylated proteins as ligands for anti-Gal

To ascertain the anti-Gal binding affinity of Lp(a) vis a vis those of other plasma O-glycoproteins, the latter were isolated by affinity chromatography on immobilized jacalin and compared to apo(a) in anti-Gal binding activity measured in terms of percentage increase in fluorescence of anti-Gal-FITC. Figure 6 shows the significantly higher affinity of apo(a), compared to the same mass of any other plasma O-glycosylated protein for anti-Gal indicating that Lp(a) is the prime candidate as affinity maturation ligand during anti-Gal synthesis.

Anti-Gal is IgG dominated, more so in high titer/small size Lp(a) individuals

While most carbohydrate-binding natural antibodies are T-cell-independent for their synthesis and are IgM-dominated,[24] exceptions are antibodies to lipid-associated antigens which engage T-cells as well.[24,25] The characteristic feature of T-dependent antibodies is their domination of IgG[25] rather than IgM. Responses of purified microplate-coated anti-Gal samples to anti-IgG and IgM were compared between low Lp(a) plasma group and high Lp(a) plasma group (16 each). Result (Figure 7) showed that while anti-Gal from both groups was dominated by IgG, Ig G domination over IgM was significantly higher in high plasma Lp(a) concentration/small Lp (a) group than in low plasma Lp(a) concentration/larger Lp (a) group (P value $= 0.0014$).

Small Lp(a) phenotype did not significantly increase specific activity of anti-β-glucoside antibody

Specific activity of a non-anti-Gal human plasma antibody, viz anti-β-glucoside antibody (ABG) was measured using cellobiose-conjugated trypsin inhibitor (TIC) in polystyrene well-coated form for antibody capture in the procedure described for specific activity assay (methods). Small Lp(a) phenotype in high plasma Lp(a) individuals was not accompanied by a significant increase in the specific activity of ABG over that of large Lp(a) individuals (Figure 8; $n = 6$ in each group; P value $= 0.402$) indicating that STPS, though a surrogate ligand for ABG[18] is a poor effector of affinity maturation of this antibody. This is also

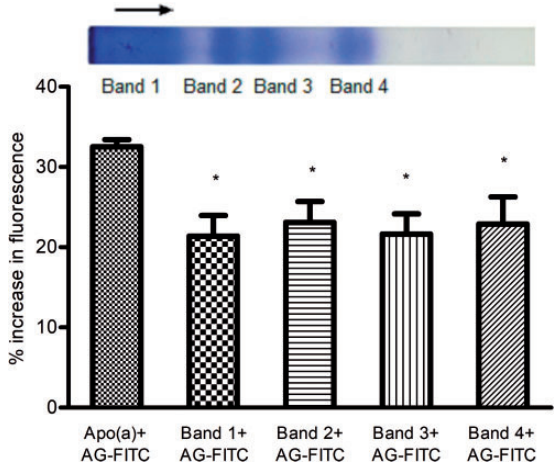

Figure 6. Comparison of apo(a) with other O-glycan-rich glycoproteins as ligands for anti-Gal. Apo(a) was prepared by DTT reduction of plasma lipoproteins as described in methods. Purified apo(a) or other O-glycan-rich glycoproteins isolated from different bands of gel (4 μg in 75 μL PBS) were incubated with anti-Gal-FITC (3 μg in 25 μL PBS) for 18 h at 4°C. Volume was made up to 300 μL with PBS just before fluorescence measurement. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on addition of other ligands. Mean ± SEM of effect of glycoproteins from six plasma samples shown. *P value < 0.05. (A color version of this figure is available in the online journal.)

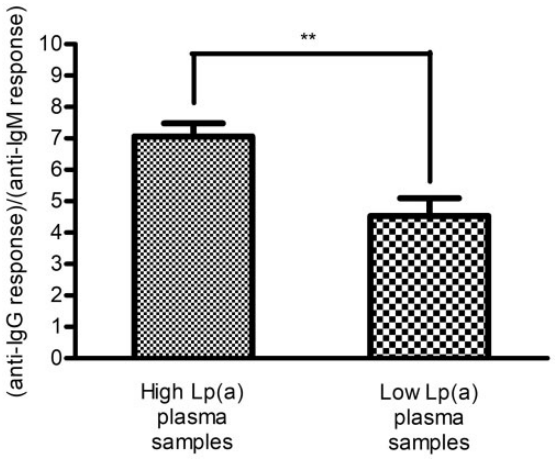

Figure 7. Anti-Gal IgG and IgM levels in low and high Lp(a) plasma samples. Polystyrene wells were coated with TIM (1 μg/well), blocked and incubated with 50 times diluted plasma in 200 μL PBS for 2 h at 4°C followed by probing with 200 μL HRP-conjugated anti-human IgG and IgM separately (1.5 μg/mL in PBST) and bound HRP was assayed. Mean ± SEM of 16 plasma samples. **P value $= 0.0014$.

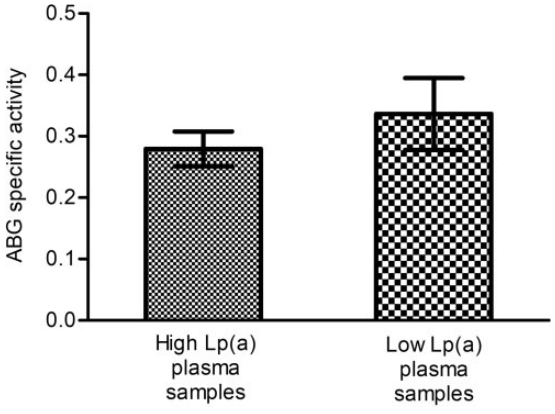

Figure 8. Specific activity of ABG in high and low Lp(a) plasma samples. The specific activity values of ABG antibody isolated from plasma with high and low Lp(a) concentrations were compared. Mean ± SEM of six plasma samples in either group. P value $= 0.402$.

evident from the absence of IgG domination in this antibody.[19]

Discussion

The number of non-covalently adhering (adduct) LDL molecules[5] attached to each Lp(a) molecule increased with the size of the apo(a) subunit of the individual so that Lp(a) free from adduct LDL was undetectably low in very large Lp(a) molecules.[11] Negative charge carried by the sialic acid moieties at the terminals of O-glycans in apo(a) subunit mediated the attachment of LDL through the positively charged amino acid residues of the apoB chain in the latter.[26] This conclusion was supported by the sharp drop in noncovalent attachment of LDL to Lp(a) following desialylation of the latter (data not shown). Nevertheless, this occupation by LDL is likely to obstruct the approach of anti-Gal, mostly IgG and much smaller than LDL, to the O-glycan-rich region preparatory to accommodating the underlying STPS at the binding site of the antibody. In support of this assumption, the O-glycan-specific lectin jacalin also blocked recognition of Lp(a) by anti-Gal.[4] Further, LDL-mediated blocking of anti-Gal binding to apo(a) subunit which was reflected in the drop in apo(a)-mediated increase of antibody-associated fluorescence, was more pronounced in high molecular weight apo(a) subunits than in the same number of low molecular weight apo(a) subunits (Figure 2) apparently due to a larger number of LDL molecules adhering to the former and blocking anti-Gal access more effectively. Confirming this assumption, the circulating larger Lp(a) molecules, despite possessing much longer O-glycosylated STPS-rich regions, were significantly less efficient in binding to anti-Gal than the same number of smaller Lp(a) molecules (Figure 3) obviously due to adhering LDL molecules whose number increases with Lp(a) size.

This negative correlation of size of physiologically available Lp(a) with its efficiency as an anti-Gal ligand in in vitro studies was also reflected in the ligand-binding affinity of the antibody in vivo as specific activity increased with decreasing Lp(a) size with a convincing correlation coefficient and P value. In further confirmation of this effect, plasma Lp(a) concentration which increases as Lp(a) size decreases showed an even more significant positive correlation with anti-Gal specific activity. These results suggested that Lp(a) which differs widely among individuals in size and thereby in antigenicity towards anti-Gal is an ideal autologous antigenic modulator of anti-Gal activity by interventions that may include Lp(a)-mediated affinity maturation of the antibody. Increasing adduct formation and a steep decline in adduct-free Lp(a) as Lp(a) size increases[11] underlined the role of adduct LDL as an attenuator of this function of Lp(a).

Besides being the most reactive among plasma O-glycoproteins towards anti-Gal, another factor that favors a role for Lp(a) as an affinity maturation antigen for this antibody is its lipid component which could act as an adjuvant.[27] Lipoproteins had been shown to become less antigenic following removal of their lipid components.[28] Notably glycan-specific antibodies to glycolipid antigens were shown to be T-cell-dependent.[24] Several human anti-carbohydrate antibodies other than anti-Gal have been shown to be T-cell-dependent and isotype-switched.[25] Sustained presence of antigen had been shown to result in up to 10 times more antibody production and affinity maturation.[27]A factor that could prevent the primate immune systems from recognizing Lp(a) as a 'self' antigen entitled to absolute tolerance is its relatively recent evolutionary origin, having emerged only about 33 million years ago.[29] Incidentally, anti-Gal synthesis along with suppression of corresponding epitope also began in these animals around the same time (~ 35 million years ago).[30]

Confirmation of elevated expression and/or exposure of MUC1 in cancer spurred efforts towards developing vaccines that generate antibodies targeting MUC1.[2] The reported increase in incidence of cancer associated with low plasma Lp(a) concentration[8] could be explained by the present results as a consequence of larger Lp(a) molecules accompanying the above phenotype giving rise to weaker anti-Gal molecules that target MUC1 less efficiently. In agreement with our results is the inverse association between incidence of cancer and Alzheimer's disease among populations[31] since the latter disease is marked by high plasma concentration/small size Lp(a) phenotype. In summary, results above suggest the possibility that variation in susceptibility to cancer depending on plasma concentration and size of Lp(a) operates through Lp(a)-dependent modulation of anti-Gal activity. This laboratory has initiated clinical research to ascertain whether higher specific activity anti-Gal induced by smaller Lp(a) protects from cancer.

Authors' contributions: All authors contributed to the concept of the paper. JJ and VK undertook most of the bench work. MG provided instrumental support, technical advice and protocol trouble shooting. JJ and PSA were mainly involved in manuscript preparation.

ACKNOWLEDGMENTS

We appreciate the supply of outdated plasma samples from the Department of Transfusion Medicine of this Institute.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

Financial assistance in the form of Ph.D fellowships to JJ and VK from our home institution and the University Grants Commission, India respectively is gratefully acknowledged.

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(Received April 2, 2019, Accepted May 14, 2019)